

## Retreat from particle physics?

*The Kendrew committee has given the expected opinion on whether Britain should pull out of high-energy physics. But was it asked the right question?*

THE Kendrew committee has had a thankless task in deciding whether Britain could continue to afford high-energy physics. Its report now published (see p. 619) faithfully reflects the difficulty of reaching a clear decision on such a complicated issue. The presenting symptom of the trouble that has arisen is that British spending on high-energy physics, taken entirely from the budget of the Science and Engineering Research Council (SERC), although much reduced in the past decade, is still (at £55 million) an uncomfortably large fraction of the total (£288 million for SERC in the present financial year). The consequences for the general support of science in Britain have been all too clear in the past few years. Good projects even in basic physics have languished for lack of funds and even, latterly, because those best qualified to execute them have been persuaded by the general climate that they had best try their luck elsewhere. Yet, curiously, it remains a fact that British spending on high-energy physics is not entirely out of line with what is spent by the other members of the European Organisation for Nuclear Research (CERN). As a fraction of gross national product, British spending on high-energy physics is less than that of France, Italy and West Germany but greater than that of Austria, the Netherlands, Norway and Greece. So why is it that Britain, for the time being at least, is the only member squealing about the cost?

There are some extenuating circumstances, of which the chief is the curious way in which the British government stolidly refuses to guarantee the costs of international collaborations against the effects of currency fluctuations. Occasionally, there may be special concessions from the Treasury, but more frequently there are not. The result is that research councils locked into international agreements for collaboration that have the force of international treaties must raid their provisions for domestic spending whenever sterling moves against them. The Kendrew committee has rightly asked that the British government should follow a more generous policy towards the research councils' difficulties in this regard. Indeed, it could well have made the point less temperately. For how can the government expect the research councils to plan carefully for the future, while following the rule that funds cannot be held over from one year to the next, when they can never know when it will be necessary to scrape the barrel for an unbudgeted few million pounds or so?

### Structure

A more serious problem is structural, and particularly concerns SERC, which differs from other research councils in that its function is exclusively to support research in universities and polytechnics, and to provide the necessary central facilities. This is the spirit in which, in more fulsome days, SERC provided not only the annual contribution to CERN but domestic high-energy

physics laboratories (long since converted to other purposes) as well. The trouble with high-energy physics is that even the reduced costs of the present operations are too large a proportion of the budget to be easily digested, but this is not the only hostage to fortune that SERC has given. Who can say whether the cost of maintaining SERC's spanking new observatories, on Hawaii and at La Palma in the middle of the Atlantic, will not become some years from now the kind of embarrassment that CERN seems now?

### Commitment

The truth is that SERC's total budget of £288 million (this financial year) is not freely disposable income in the true sense. Large parts of it are committed in advance, not merely for the payment of annual instalments of research grants awarded in previous years and for maintaining central facilities and their staffs, but also because SERC has been persuaded, in what seems to have been the national interest, that it should support with its own funds substantial government initiatives, for example, information technology, and at the same time increase support for engineering research at universities. The CERN subscription, however onerous, is only one of the several commitments that explain why SERC has had so little to spend on the core of scientific research. So the Kendrew committee might reasonably have argued that it might have been spared the trouble of existing if SERC, over the years, had more zealously managed its affairs for flexibility. Somewhat delphically, all that Kendrew says is that "the organization of science in Britain should be rationalized so that funds available are used more effectively". That, presumably, is what people have been attempting for decades past.

The course of action now suggested is an awkward compromise. Nothing much will be saved in the next three years or so, although there will no doubt be a flight by graduate students from high-energy physics into other fields. Since there is very little chance of the government relenting, and allowing a substantial increase of the funds available for civil science, SERC will continue to be unable to support the projects now sent away begging for at least that long. And even if the negotiations with CERN are successful (which is improbable), there will not be much extra in the kitty — perhaps £15 million a year seven years from now, a mere 5 per cent of the annual budget. In the circumstances, it would have been better that the Kendrew committee should have bitten hard on the bullet put between its teeth, should have counted the real British moral obligation to CERN as a small consideration compared with the survival of British science, and should have plumped for withdrawal at the end of 1986 (the earliest date possible). It could have added that it would have preferred to have been asked a different question, and might even have formulated some of the more intelligent questions that need answering. But that way, at least, there would have been an early prospect of relief from the present crisis.

### Ructions

But would not such a course have been a recipe for ructions? Of course, Llewellyn-Smith (see p.619) is entirely right in most of what he has to say about the consequences for CERN, and for

## Biological manuscripts

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the British reputation in European collaboration, of even the compromise course of action now recommended. Outright withdrawal would have caused a bigger row, but one not qualitatively very different. Moreover, such a decision would have laid the trouble squarely where it belongs, with the government whose niggardliness has brought about the crisis of the past several years. Indeed, it is entirely possible that if the British government as distinct from SERC were faced (as it may be still) with the task of engineering withdrawal from CERN, it would find the task politically unpalatable.

Whatever happens now, the Kendrew committee has demonstrated vividly that something must be done about the condition of academic research in Britain. One of its most compelling passages is the account which it has gleaned from the public grant-making bodies of the projects the research councils are unable to support for lack of funds. This is also the theme of last week's report from the Advisory Board for the Research Councils, published early so as to inform last Friday's debate in the House of Commons (see p.623). (By all accounts, Kendrew would have been similarly dealt with if only the Department of Science and Education had had an extra word-processor.) Quite why that occasion took place is still far from clear. The initiative was that of the Secretary of State for Education and Science, Sir Keith Joseph, but on this occasion the apostle of management by cash limits spent much of his time reciting the difficulties attending British academic research, the flight of people, for example. Was the debate Sir Keith's own way of acknowledging that his critics have won the day, and of recruiting their help in talking down his colleagues in the government?

However that may be, it seems not to be fully appreciated that an urgent remedy is required. Some good ideas have always been wasted because there are no funds to back them. What matters now, in Britain, is the sheer volume of talent going to waste. Even the good ideas that, with luck, find backers are often supported with such economy that their success is jeopardized. Last week's advisory board report gives several examples of projects with industrial importance which are less successful than they might be for lack of adequate support. Is it any wonder that, in these circumstances, people's spirits have evaporated? Or that those not yet entirely demoralized think first of taking their skills elsewhere?

## Yearning

The simple solution is that the government should find some extra money, on a strictly short-term basis, to see that projects of outstanding quality (and their authors) should not be lost during the painful but necessarily cumbersome reorganization of the research enterprise now in prospect. An extra £100 million spread over the next three years would do an immense amount to help. If the government is afraid of distributing such a bonus among the research councils in the usual way for fear of giving them ideas above their station, it might think of distributing the money by some different mechanism. It would be by no means unreasonable or inappropriate that there should be a national competition for shares in the central fund. If the government's yearning for industrial collaboration is seriously intended, grants from such a special fund might be made contingent on matching contributions from outside. Is that modest request too much to ask?

Otherwise or perhaps in any case, the research community should take what steps it can to hasten the reorganization of resources on which it has embarked, but which will work its way through the system only after several years. The University Grants Committee and the research councils have the common objective of concentrating resources for research on the institutions best qualified to make use of them, but the process will not begin until next year. Why wait that long? And why, in the interests of fairness, contemplate a long period of transition from the present indiscriminate distribution of resources to a less egalitarian system? Why not move faster, recognizing that the pursuit of fairness may be a luxury when the danger is that the quality of British research will be irreparably damaged? □

# The logic of Europe

*The plan to turn Europe into a true customs union by 1992 deserves a welcome.*

At long last, there is a chance that Europe will become the integrated economic entity of which the founding fathers dreamed thirty-five years ago. Or, at least, the member governments (now twelve, with the accession of Spain and Portugal) will be compelled at next month's annual summit meeting at Milan to come off the fence and to make plain what kind of Europe they wish to see emerge in the remainder of this century. Ordinarily, governments struggle to avoid answering questions of such a general character. The need now to face them arises because the European Commission, the executive branch of what passes for the government of the European Communities, has produced an unexpectedly radical reply to a request last year from member governments for an outline of the steps that would have to be taken if internal customs posts were to be abolished. What the Commission says is simple. There is no reason why customs posts should not be swept away, but it will first be necessary for European governments to make more similar the domestic taxes they impose on the sale of goods.

There is the rub. Over the years, European governments have allowed the accumulation of a bewildering variety of indirect taxes on goods as different as alcoholic drinks, tobacco products and commodities considered to be luxuries, motor cars included. Then, membership of the European Communities has compelled the development of a novel system of indirect taxation, called value-added tax (VAT), which is levied at different rates on different classes of goods by the several members of the Communities. The continued existence of customs barriers within Europe is partly a consequence of this patchwork system of indirect taxation. The Commission now proposes that member governments should be constrained in their freedom to levy indirect taxes. During a transitional period, they will be expected not to allow the rates of tax to range outside prescribed limits. The Commission says that it should be possible to arrange that everything could be ready for the abolition of internal customs posts by 1992, which is asking a great deal. The immediate objection, of which much will be heard at Milan, is that the member states are not ready to relinquish such a visible sign of national sovereignty as their freedom to fix domestic taxes.

Pragmatically (which is in itself unusual but welcome), the Commission does what it can to defuse this argument by pointing out that the differences between the taxes levied on goods at the point of sale are not so great that their approximation during the next seven years will be impossible. And, in any case, the argument continues, the experience of federal systems such as the United States has shown that even substantial differences between sales taxes levied in neighbouring states do not send consumers scurrying across state lines in search of bargains in the shops. But there is an important issue that the Commission overlooks — the possibility that member states may wish to engineer substantial changes in the balance between direct and indirect taxes in their national accounts. For entirely good reasons, the government of New Zealand has already shifted the burden of taxation from income to consumption taxes (and lost the Timaru by-election at the weekend chiefly as a result). The government of Australia is planning to follow suit, while even the British government has, in previous years, talked of similar proposals. So the European Commission's proposals are more radical than they may seem. Prudent member governments will accept them — and they should — only if there is a mechanism by which general questions of taxation policy can be raised and decided on a European basis in the decades ahead. But that will imply a substantial step along the road to a federal Europe, as the objectors at Milan will be quick to say. The logic of the circumstances that have now arisen, however, is that Europe can not hope to be an integrated market of nearly 400 million people without becoming at the very least what many outsiders think it is already, an effective customs union. □

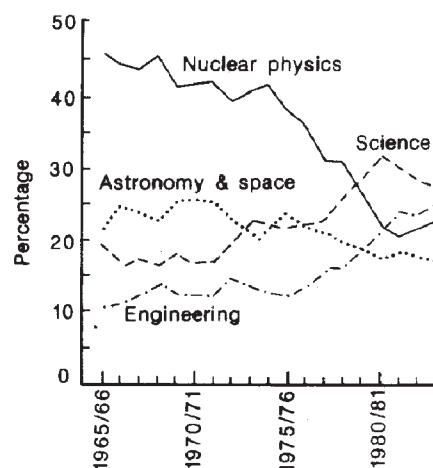


## CERN

# Kendrew takes knife to high-energy physics

THE technical consultant to the Kendrew committee on British involvement in high-energy physics, Dr Christopher Llewellyn-Smith, had repudiated the committee's chief conclusion (see *Nature* 29 May, p.266) within minutes of its formal publication earlier this week (*High Energy Particle Physics in the United Kingdom*). Of the recommendation that Britain should seek a reduction in its contribution to CERN, the Geneva high-energy physics laboratory, of "at least 25 per cent by 1991", Llewellyn-Smith says that the "unsubstantiated assertion" that such a reduction could be attained without detriment to CERN's position is, simply, "false".

That the Kendrew report would be contentious has always seemed probable. In the event, however, the report is a model of clarity, which should comfort both its supporters and its critics. Briefly, the conclusions are that Britain should remain a member of CERN "on the present basis" until 1989, but that it should continue beyond that time only if that can be done "at significantly lower cost". The timing is determined by the construction programme for the new electron collider LEP.



The committee notes that British scientists continue to make important contributions to high-energy physics and that the subject is at present at an exciting stage, but it also says that British spending on high-energy physics is too great a proportion of the present or "any realistic" budget of the Science and Engineering Research Council (SERC). It recommends that modest economies should be made as soon as possible, principally in the budgets of groups working on experiments at CERN, does not expect these to be substantial before the financial year 1988-89, but would expect them to increase to "at least 25 per cent" by 1991.

The committee says that the proposed reduction of the CERN budget would be an "attainable adjustment" that would "still allow the laboratory to maintain itself at a world class standard albeit at a reduced pace of development". The proposed reductions of domestic expenditure would entail "a significant decrease in the size of the experimental community" (now estimated to consist of 400 senior people and 240 graduate students), but the committee says that it should be possible to arrange for an orderly contraction while maintaining an "appropriate" British contribution to the field.

The report argues that the financial circumstances of the United Kingdom which have led the committee to its conclusions "may not be unique" among CERN members, that "the overall level of expenditure on the subject is too high" and that "it would not be counter to the long-term interests of the field" if the pace of development were reduced "worldwide and not merely at CERN".

The committee acknowledges that the total budget of CERN has decreased by 20 per cent in real terms over the past decade, and that British spending on high-energy physics has declined during the same period "from what was an extraordinarily high level". It also says that there is insufficient support for first-class research proposals both in high-energy physics and in other areas.

The report suggests that there is at present little room for manoeuvre within the British budget, pointing out that there would be no point in remaining a member of CERN if it were not a first-class laboratory and if domestic funds were insufficient British physicists to make full use of it. The report also says that there is a "strong moral obligation" on the United Kingdom to remain a member of CERN until LEP is finished as well as to continue development of the three particle detectors being built for use with LEP.

The Kendrew committee therefore argues that the British delegation to CERN should press for an immediate review of the financial structure of the CERN laboratory, with the objective of reducing costs after 1989. Allowing for both domestic savings and reduced expenditure at CERN, the committee calculates that it should be possible to save a total of \$55 million over the next seven years, or an annual \$13.8 million (at present prices) in 1992-93 and succeeding years. The Kendrew committee asks that the British government should help to avoid future difficulties by arranging that the budgets of the research councils will

not be further eroded by inflation, and that the research councils should be insulated from fluctuations of exchange rates.

The committee goes on to say that even the saving likely under its proposals will not "in itself solve the financial problems of other areas of science", and that extra funds will be needed if the British research base is to meet "the challenges of the next decade".

Formally, the report is a recommendation to SERC and the Advisory Board for the Research Councils, on whom will fall responsibility for deciding what should be done. The most likely next step is that negotiations will be opened with CERN on the proposed reduction of the budget. Most probably, there will be no biting of bullets unless and until those negotiations seem about to fail. □

## Informed dissent

THE dissenting opinion from Dr Christopher Llewellyn-Smith, reader in theoretical physics at the University of Oxford, is strong meat. It begins by saying that the real issue facing the whole of British science is "the erosion of our superb scientific tradition" by the inadequacy of funds. Llewellyn-Smith says that he informed the Kendrew committee of his dissenting opinion before making it public.

Among Llewellyn-Smith's objections are the following.

- The demand that the British contribution to CERN should be reduced by 25 per cent implies a similar reduction of the total budget.

- Given that salary costs at CERN are roughly half of the total budget, reducing the budget by a quarter would require either a disproportionately larger cut of scientific activity or a reduction of the staff employed (which would not yield immediate savings because of the high cost of redundancy).

- It is inconceivable that the necessary two-thirds majority for a 25 per cent cut in the CERN budget could be found among the present membership, for which reason the committee's main conclusion "implies withdrawal . . . the committee fails to face up to this consequence."

- The suggestion that the proposed domestic reduction of 25 per cent could be orderly "is ludicrous"; because of the long timescale of high-energy physics experiments, the cuts would fall on those whose projects are about to be completed.

- As recently as 1981, SERC offered the opinion that the budget for nuclear physics should remain constant, since when high-energy physicists have made long-term commitments.

- The recommendations in the report would seriously damage particle physics, would damage the credibility of the United Kingdom as a collaborator in science projects and yet would make only a small contribution to the "solution of the crisis in science funding". □



## Sandia Labs

## Looking to the future

*Albuquerque, New Mexico*

A CARTOON circulated recently at Sandia National Laboratory shows a wizard complete with pointed hat gazing intently into a crystal ball; a sign on the door in the background reveals the location to be the head office of the Future Options Group (FOG) an 8-man team of defence scientists which spends its time trying to do just that. Glenn Kuswa, manager of the FOGs (as they are known) for the past 10 months, admits that the mysterious-sounding group of futurists that he leads is a fair target for cartoonists, but he insists that the group is taken perfectly seriously by Sandia's senior management. In a national laboratory with primary responsibility for defence, future-gazing is a serious business.

FOG was formed to advise on long-term planning at Sandia only three years ago, but has already had a significant impact on both the weapons development and the basic science at Sandia. Its concern about the importance of high-speed computing, for example, led to a new laboratory division devoted to computer architectures. Sandia, in addition to carrying out contract research (mainly for the Department of Energy), is charged with anticipating future defence needs, and several of its major research efforts, such as sub-micrometre semiconductor technology, were initiated by the laboratory management.

While the laboratory's primary mission is still research into nuclear weapons, the scope of this work has broadened considerably in recent years, so much so that the laboratory recently had to be reminded by Washington that biological research, at any rate, could not be considered part of its mandate. More and more projects are carried out in collaboration with commercial companies, and the number of staff has grown to match. Sandia now employs more than 8,000 people, a figure that is not expected to increase further.

FOG is the creation of an informal group of laboratory managers seeking a mechanism for looking beyond the normal course of events, pondering how Sandia should anticipate the future.

The group started by concentrating on strategic defence, and gave advice on the subject to the White House long before President Reagan's famous star wars speech of March 1983. Missile defences continue to take up a large part of the FOGs' time. Electrothermal guns might, according to Kuswa, be capable of accelerating projectiles to even greater velocities than the rail guns now being intensively investigated for the Strategic Defence Initiative: the physics of impacts at extremely high velocities and their implications for weapons design are one focus of

the FOGs' attention.

So far, most of FOG's divinings have been delivered as briefings to Sandia's senior management, with some informal contacts with outside bodies such as the Congress's Office of Technology Assessment. Kuswa thinks FOG has played an important, if little-known, role in fostering communications between different factions at Sandia, and wants to see it move towards producing more formal written reports in future.

Not surprisingly, the successful maintenance of nuclear defence is counted the group's primary objective. The issues tackled range from the particular — for example, hypervelocity guns — to the general — brainstorming and arranging training courses in global security issues such as the world's energy supply.

Kuswa has thought about how the "nuclear winter" hypothesis might affect deterrence, but FOG is not yet committed to studying the topic in earnest. By and large FOGs just think, rather than do experiments, and Kuswa thinks that the group should wait until there are more data on nuclear winter before addressing it.

FOG can see further into the future of photonics and fibre optics, which might lead to revolutionary advances in computing speeds and which Kuswa says will certainly have important implications for the way high-speed data are recorded. Such matters are crucial for the strategic balance of power, and Kuswa believes computing is important enough to warrant at least one FOG member permanently tracking it. Such is the speed of progress that Sandia boasts that it has recently produced an experimental computer nick-

named the "VAX in a lunch box".

Software engineering is another topic FOG is studying; Kuswa is alert to the possibility that, despite what many see as a disappointing track record so far, artificial intelligence research could yet rock the strategic boat. In one attempt to make the future come a little sooner, Kuswa's group recently sought to apply expert systems, a spin-off of artificial intelligence research, to some of the laboratory's work. A retired employee had his expertise on the electrical wiring and connectors of nuclear warheads programmed into an expert system for the laboratory's future use. Kuswa hopes to persuade Sandia to make further use of expert systems for similar tasks in future.

A related subject is the likely availability of custom integrated circuits. Whereas circuit design is today still the province of the expert, the increasing sophistication of computer-aided equipment suggests that before too long the design and manufacture of a high-performance integrated circuit engineered for a specific task will be within the reach of most engineers. Contrary to FOG's usual approach, the group recently staged a demonstration of the production of specific integrated circuit.

Kuswa would like ultimately to establish a database of all military hardware, as a resource for studies of what the Department of Defense's shopping list is likely to look like in future (future bombing "platforms", including unmanned ones, are the subject of a current study). This, however, has proved difficult and is unlikely to be accomplished soon. In the meantime, there are plenty of other subjects to think about, and Kuswa can apparently see far enough into the future to know that FOGs are going to be in continuing demand. "We have," he says, "earned our keep."

Tim Beardsley

## Cell lines jump US customs

*Washington*

THE US Department of Agriculture (USDA) has agreed to simplify and streamline its quarantine rules for the import of cell lines. Biotechnology companies have been complaining of delays of as much as a year while tissue cultures and tissue-culture products await testing at USDA's Plum Island quarantine facility. Industry sources say they hope the new procedures will cut that time to two months.

USDA's quarantine rules apply to imports of any materials that could contain foot-and-mouth disease virus and other livestock diseases not already present in the United States. Thus cell lines prepared with bovine serum or the enzyme trypsin (which is derived from cows and pigs) come under the quarantine. The safety test involves inoculating a steer, waiting 4-6 weeks and analysing a blood sample.

During negotiations between USDA and

representatives of the biotechnology industry on speeding up the process, it became clear that most of the delays were bureaucratic. Companies would, for example, be sent a bill for a test that had to be paid before testing would begin. Under the new procedures, companies will be able to establish deposit accounts with USDA so that the tests can be run as soon as products arrive at Plum Island.

The new rules will not, however, provide exemptions from the strict testing requirements that the industry had been seeking in respect of materials to be used only in clinical applications or *in vitro* diagnostics. The industry argues that so long as such materials are disposed of properly, there is virtually no chance of livestock being exposed. USDA officials say that discussions with the industry on this issue will continue.

Stephen Budiansky

## Polish universities

## Reforms threaten independence

POLAND'S Main Council for Science and Higher Education, the representative body of the university community which serves as an intermediary between the universities and the Minister of Science and Higher Education, stands to lose most of its powers in the planned reform of the 1982 Higher Education Act. Since the discussions on reform began last autumn, the Main Council has resolutely opposed the proposals which would effectively annul the university autonomy won during the Solidarity era. A vote taken on the proposed changes early in March showed only one member of the council in favour of the changes, with a handful of abstentions. Now, a new draft of the proposed reforms, which reached us last week, threatens the status of the Main Council itself.

The 1982 Act established the Main Council as the supreme elected organ of the university community. It has seventy members, fifty from the universities and colleges under the ministry and the rest from the medical, physical training, art, drama and maritime colleges. The council has power to decide on the direction of research and the organization of studies, to set the conditions for the awarding of academic degrees and has the right of consultation with the minister on a wide range of financial and administrative matters. The original version of the reform strengthened ministerial control and disciplinary powers over the universities, bringing the "students' self-government committees" within the framework of the Party youth organizations, but the role of the council was left virtually unaltered.

With the Main Council resolutely opposed to the proposals, however, the government clearly decided to think again. Of late, the issue has no longer been in the hands of the minister of science, Dr Benon Miskiewicz, but had been handed over to the government's Committee for Social and Political Affairs, under Deputy Prime Minister Mieczyslaw Rakowski. The new draft which has emerged would impose considerable restrictions on the Main Council. First and foremost, the members of the Main Council would no longer be elected, but instead would be "appointed by the chairman of the Council of Ministers at the request of the Minister of Science and Higher Education" (Art. 18.1). "Directions of study" will now be decided not by the Main Council but by the minister (Art. 8.2). And, under the greatly revised Art. 2, the Main Council will no longer "decide at the request of the Minister" but only "give an opinion" on "possible and definite plans for the development of higher education". The same article does, indeed, leave decision-making on research within the universities and in the competence of the Main Council, but with the proviso that its decisions

must be within the framework of the central economic plans. On matters affecting the terms of employment of academic staff, the minister will decide "after hearing the opinion of the Main Council" instead of, as at present, "in agreement with the Main Council".

The latest version of the reform will therefore reduce the Main Council to, at best, a powerless talking shop for the universities and, at worst, a rubber stamp for

the decisions of the minister and the economic planners. During a special plenary session of the Central Committee of the Polish United Workers' Party last month, General Jaruzelski reiterated the official viewpoint, that the universities cannot any longer be allowed to be the breeding ground of political opposition, but must be more closely incorporated into the socialist structure. This triggered nationwide student protests, as a result of which Rakowski and Miskiewicz found it necessary to assure university rectors that the reforms would not be made an excuse for a political purge.

Vera Rich

## Electricity supply

## Cross-Channel power games

FRANCE will begin pumping 1,000 MW of its cheap nuclear electric power across the English Channel into the British grid later this year, when the first stage of a new 2,000-MW undersea link is completed.

The new cables — four of them — run from Les Mandarins, south of Calais, to Folkestone. They will increase the French-British power linkage more than tenfold, from the present 160 MW transferred along a line between Dungeness and Boulogne mainly to even-out peak demand on each side of the Channel.

But the new link will open in two 1,000-MW stages. And while the English utility, the Central Electricity Generating Board (CEGB) and its French partner, Electricité de France (EDF), have reached agreement on the first stage, for France to supply Britain with cheap power, the use of the second 1,000 MW is still in dispute.

On its side, EDF would like CEGB to buy another 1,000 MW of "baseload" continuous power at French marginal supply prices, which EDF is quoting at attractive rates. This is not surprising, as within a few years the French electricity supply will

be more than 70 per cent nuclear. Nuclear reactors are hard to turn on and off, so the responsiveness of the French network to demand fluctuations has been decreasing, despite efforts to make French pressurized water reactors more variable.

CEGB, on the other hand, in considering a second 1,000 MW from France, is said to be concerned about its agreements with the British National Coal Board (NCB), now recovering from its year-long coal strike. CEGB is by far the coal industry's biggest customer, and is contractually committed to buying large amounts of NCB's coal. CEGB cannot buy too much French power without endangering those agreements, and the British coal industry, it is claimed.

Or, to put it differently, it seems that while 1,000 MW of French electricity is convenient enough to make a sharp point to the government and NCB about the value of nuclear power — and a warning that coal prices had better be kept down — 2,000 MW from France might be contractually uncomfortable.

Robert Walgate

## CSIRO on the right rails?

## Canberra

THE Australian Minister for Science, Mr Barry Jones announced last week the appointment of Dr N.K. (Keith) Boardman as the next chairman of the Commonwealth Scientific and Industrial Research Organisation (CSIRO). Dr Boardman, for the past eight years a member of the three-man CSIRO full-time executive and formerly chief research scientist of the CSIRO Division of Plant Industry, will take the reins for nine months in the first instance, beginning in September when the present chairman Dr J.P. (Paul) Wild retires. Mr Jones also announced the appointment of two new part-time members of the executive, Dr Adrienne Clark, a Melbourne University botanist, and Dr Kevin Foley, chairman of the Australian Industrial Research and Development Board. Like that of Dr Boardman, their appointments are

for nine months pending the outcome of a series of reviews into government-sponsored science in Australia being undertaken by the Australian Science and Technology Council, in the course of which CSIRO is only the first research establishment to come under scrutiny.

The conclusion of the CSIRO review is meant to coincide with Dr Wild's retirement. A former chief research scientist of the CSIRO Division of Radiophysics and 1980 Royal Medallist of the Royal Society, Dr Wild has personally lifted the public profile of CSIRO through highly-visible and commercially-orientated work on the new aircraft landing system Interscan and, more recently, his plan to link Australia's two largest cities with the national capital Canberra using trains operating at speeds of up to 350 kilometres per hour.

Jeffrey Sellar



## Arianespace

## Europe's launcher cashing in

ARIANESPACE, the French-based company that sells the successful European space launcher Ariane, has tripled its turnover compared with projections made when the company was set up five years ago, according to company president Frédéric d'Allest last week. Bursting with enthusiasm, and apparently happy to challenge competitors in the United States and anywhere else, d'Allest said Arianespace has 35 satellites and FF7,000 million (£600 million) on its order books.

"There has been a very dramatic increase of activity" and "we at Arianespace are the only launch agency that can balance its accounts", d'Allest said. Ariane-space was planning three launches a year and now is planning a firm six, with up to eight a year possible.

A further new launch pad, ELA 2, will be inaugurated at the Kourou launch site in French Guyana, north of Brazil, in October. At only five degrees of latitude from the Equator, compared with Cape Canaveral's 28 degrees, the site has the benefit of greater natural launch velocity (from the Earth's rotation).

At the same time, d'Allest dismisses all competition in launch vehicle manufacture. In the United States, attempts are being made to commercialize the "obsolete" Atlas Centaur and Thor Delta rockets. But there will be no significant improvement in the technology of expendable launchers in the United States, he claimed. For, d'Allest argues, no commercial company can bear the costs of launcher development while the US government is unlikely to make a substantial

contribution to the development of expendables for fear of undercutting the economics of the space shuttle.

Meanwhile Ariane development goes on apace, he said. The penultimate Ariane-1 should launch the Halley probe, Giotto, Ariane's first interplanetary mission, on 2 July, while the slightly bigger Ariane-3 has become the main Arianespace workhorse. Ariane-4, with strap-on liquid and solid-fuel boosters, designed for a new generation of telecommunications satellites, will be launched in mid-1986 and enter commercial service the following year.

After Ariane-4, a completely new design of Ariane will take over, a design using HM-60 cryogenic engines now under development by the French national space agency CNES. This "Ariane-5", capable of launching the bigger communications satellites being developed, will carry Arianespace from 1995 to the end of the century.

Arianespace could face some competition from Japan's H2 launcher, but H2 is too small, more comparable with Ariane-4 than Ariane-5, said d'Allest. Moreover, Japan is constrained like the United States by the northern location of its launch site.

The problem with the Soviet Union, a potential competitor, is that its launch sites are under military control and very tight security, so very little information emerges on launch technologies, communications facilities and so on, too little to make possible fruitful cooperation with a commercial client. In China, the Long March 3 rocket, at the testing stage, is similar to Ariane-1. Chinese technology will develop, but like Japan, China is hampered by a northern launch site. And the painful West German experience in the private OTRAG venture, which attempted, unsuccessfully, to use ultra-cheap technology and a Zaire launch site, showed that there was no easy route to space. d'Allest acknowledges that fibre optics may become competitive with communications satellites on certain trunk routes, but considers that there will be enough launcher business left to keep Arianespace healthy.

That leaves Arianespace, which has taken 48 per cent of the world commercial satellite market since 1981. "in the clear", given that the US space shuttle has proved expensive. The company's objective, according to marketing manager Jean-Claude Bichet, is to take one third of the market, which is to be found 40-50 per cent in the United States, 18-20 per cent in Europe, 10 per cent in the international bodies Inmarsat and Intelsat, and 20 per cent in the rest of the world. Arianespace hopes soon to be launching 8 to 12 commercial satellites a year.

Robert Walgate

## Exhaust emissions

## No agreement in Europe

Brussels

VEHICLE emission limits announced two weeks ago by European environment commissioner Stanley Clinton-Davis are expected to receive a very cool reception from the ten environment ministers when they meet in Luxembourg on 25 June, which could yet again delay a decision to control car pollution.

Last March, environment ministers of the European Community had agreed to stagger deadlines for the introduction of new emission standards according to engine capacity, and had asked the European Commission to provide exact figures for emission limits for carbon monoxide (CO), hydrocarbons and nitrogen oxides (HC and NOX) and for nitrogen oxides on their own (see *Nature* 314, 304; 1985). These figures, it was agreed, would achieve an environmental effect equivalent to that reached in the United States, and would allow the use of lean-burn technology in the case of the medium category of car (1.4-2.0 litre).

But the limits suggested last week for medium cars (30 grams per test for CO, 8 g/t for HC plus NOX and 4 g/t for NOX) seem to be unobtainable with lean-burn technology. When differences between the European and US test cycles are taken into account, the suggested limits are stricter than the standards applied in the United States and could remove 20 per cent of present large cars from the roads.

Several countries consider these new figures unrealistic and feel they do not correspond with what was agreed at the March council. The United Kingdom had suggested 35 g/t for CO, 12 g/t for HC plus NOX and 6 g/t for NOX, while Italy and France, which have carried out their own tests in the meantime, maintain that 4 g/t for nitrogen oxides is not achievable except by using the catalytic converter.

Mr Clinton-Davis is determined to take a firm stand. His aim is to see NOX emissions in the Community cut by half from 3 million tonnes to 1.5 million tonnes.

But the Commission's proposals are unlikely to please those countries seeking a more flexible approach. Furthermore, they will once again put West German interior minister Friedrich Zimmermann in a difficult position. At the last council, he obtained Community approval to go ahead with plans to introduce national tax incentives for clean cars, but these incentives have to be tied to clear-cut emission standards. If Community ministers cannot agree, Zimmermann will either have to rely on US standards when the incentives come into effect on 1 July or continue to hold up the project, which could further adversely affect an already depressed car market.

Anna Lubinska

## Rocketing insurance

ARIANESPACE may have to wait a year before it can announce reductions in the insurance premiums for satellites launched on Ariane, even though these premiums have reached punishing levels after satellite losses on the space shuttle (see *Nature* 6 June, p.447). So said M. Charles Bigot, director-general of Arianespace, who was in London last week with his president, Frédéric d'Allest, to discuss, among other things, new insurance terms with City of London brokers.

"We had hoped to announce new terms at the Paris air show a few days ago", said Bigot, but the problem has proved more difficult than expected. "The insurance companies are treating all launchers as if they were the same kind of car", Bigot complained. But Arianespace is not going to fight dirty and use French government pressure, as it could now that most French sources of finance are nationalized. "This is a commercial question and we shouldn't mix it with politics", he said.

Robert Walgate



## British science

## Commons debate cuts little ice

LITTLE blood was drawn, and no concessions won from the British government, during last Friday's debate on science in the House of Commons. But the government had agreed to the demand of Dr Jeremy Bray, the Labour Party spokesman on science, that this year's advice from the Advisory Board for the Research Councils (ABRC) should be published for the debate; it asks that the government should give the research councils £85 million extra over the next three years and wrings its hands over the rate at which younger scientists are leaving Britain.

Sir Keith Joseph, Secretary of State for Education and Science, admitted at the outset that a "significant" part of the science budget has had to be spent on activities other than research, but insisted that the government has protected the budget in gross terms. This assertion was disputed by many members of parliament (MPs) during the debate (see also *Nature* 23 May, p.271), for example by Dr John Marck, who pointed out that the Agricultural and Food Research Council (AFRC) is facing cuts of £10 million this year and £20 million next.

Sir Keith also acknowledged that the number of scientists leaving Britain to work abroad where the potential rewards are greater is a matter for concern. On the other hand, he was encouraged by the increased collaboration of the research councils (which fund much of Britain's civil research) with industry in a selective and efficient way. He applauded the reduction of the research council's long-term commitments and their consequent increased flexibility and concluded that, for British science, "the way forward must be to scrutinize carefully the priorities implicit in the present balance of funding between subject areas and to consider whether some adjustments may be needed better to serve the national interest". He promised to assess ABRC's recommendations in this year's public expenditure survey, but demanded more investment in basic science from industry, particularly in engineering.

Opposition and Conservative MPs alike demanded action from the government to keep Britain in the front line of international science research. Dr Bray said that the least the government could do is to guarantee ABRC's recommended increases. His worries about the enforced drift of scientists elsewhere were epitomized by the £25 million laboratory set up by a US pharmaceutical company for Dr Leslie Iversen, formerly head of the Medical Research Council Neuropharmacology Unit at Cambridge. Dr Bray said that the work should be available to science generally and to British companies for commercial development, rather than being "pre-empted" by a US company. He

argued for the creation of a Minister of Science, and for an annual survey of industrial research and development.

Mr Ian Lloyd (Conservative) endorsed Dr Bray's proposals but had one of his own to make as well, the setting up of an Office of Technology Assessment similar to that in the United States. This independent body would serve both houses of Parliament, would be non-political and would address fundamental issues of current interest such as Mr Enoch Powell's embryo protection bill, acid rain, nuclear power and the commercial implications of biotechnology.

ABRC has set up its own working party, chaired by Professor Mathias, to examine funding of research by the private sector and to consider how it is to be extended. Its findings will be published in September of this year.

Among many other suggestions as to how the government should support sci-

ence was that of Mr Robert Jackson MP to find money from the defence research and development budget. In 1981, Britain spent \$3,000 million on civil and \$3,256 million on defence research and development. Equivalent figures for France and West Germany in the same year were \$4,370 and \$2,591; and \$6,698 and \$646, respectively. Britain could, therefore, adjust its balance of spending accordingly.

● Mr Enoch Powell used the debate to discuss once more his bill to protect human embryos. His argument was twofold. First, he failed to find any evidence, by correspondence with this journal, scientists and doctors, that any research of the kind that would be forbidden by his bill is actually being performed, arguing that his bill should therefore be passed. Second, he suggests that those who opposed his bill were the unwitting dupes of corporate giants that stand to gain by developing contraceptive and other drugs using human embryos as experimental material. Both these arguments, as well as the appropriateness of the issue in this debate, cut little ice. **Maxine Clarke**

## US agencies

## NSF's hypothetical spree

## Washington

THE idea of a federal Department of Science and Technology, which briefly resurfaced last winter for approximately the 100th time in the past 20 years, according to a tally by the Congressional Research Service, has appeared yet again, this time in a slightly veiled form. This week, the National Science Board, the governing body of the National Science Foundation (NSF), is to receive a study of what the agency would do if its budget were to increase by a factor of three over the next five fiscal years. The study, commissioned by NSF's director, Erich Bloch, included the assumption that as well as the extra money, NSF would take over the extramural research programmes of the Departments of Agriculture, Energy and Defense.

Although last winter's proposal rapidly fizzled out and while there is little chance that a budget-deficit-minded Congress would consider such a proposal even if it were submitted, the NSF study makes clear that those in high places would like a major reorganization of US science agencies.

One often-discussed idea, less drastic than consolidating all the science agencies into a single cabinet-level department, is a merger of NSF and the National Bureau of Standards, as proposed by the Reagan administration in 1983 as part of a plan to replace the Department of Commerce with a Department of International Trade and Industry. The proposal of last winter (in a study by the Presidential Commission on Industrial Competitiveness), took that as a starting point; the commission also

considered adding in the research portions of the Department of Energy, the National Aeronautics and Space Administration and the National Oceanic and Atmospheric Administration.

Among the supporters of a cabinet-level department at that time was George Keyworth, the president's science adviser. And Roland Schmitt, the president of the National Science Board (and vice-president for corporate research and development at General Electric) said in a talk last winter at George Washington University that while he was unsure whether a Department of Science and Technology was the answer, the present organization of science agencies needed to be reexamined in the light of a failure to provide adequate support for the "science base" in the country. Schmitt said that NSF had traditionally supplied this base, which he said included the training of new scientists and engineers as well as research on new concepts too risky for industry to invest in, with help from the "mission agencies".

But now, he said, the mission agencies had become too involved with their missions to keep up their end. "Maybe what we need is not a Department of Science and Technology but an NSF that is three times the size of the present one", said Schmitt.

Last week, however, NSF officials denied that the study ordered by Bloch was anything more than an "intellectual exercise" to identify research priorities and said that NSF had no wish to take over the research responsibilities of other agencies. **Stephen Budiansky**

## Deep-sea diving

## Japan finds clams and trouble

## Tokyo

ON its second dive to the Tenryu Canyon off the Pacific coast of Japan, the French deep-sea submersible *Nautille* struck gold with the discovery of colonies of giant clams at a depth of 3,830 m, the deepest record so far of "vent communities" of organisms. But no sooner did news of the find break than *Nautille* sustained serious damage when surfacing in rough seas. Repairs to the submersible will probably take at least a month and may require the postponement of the Franco-Japanese project Kaiko until next year.

The colonies of clams were found on 6 June by Professor Claude Rangan of the University of Paris during a dive to the deep-sea fan at the mouth of the Tenryu Canyon, a steep-sided submarine valley that cuts into the accretion wedge of sediments being stacked up along the edge of the Eurasian plate as the subducting Philippine plate plunges beneath Japan. The giant clams are up to 15 cm in length and have been provisionally identified by Dr Suguru Ohta of the Ocean Research Institute, University of Tokyo, as *Calyptogena* sp., a genus of bivalve typical of the "vent communities" of organisms reported from mid-ocean spreading ridges and the subduction zone off the coast of Oregon.

According to Asahiko Taira, professor of sedimentology at the Ocean Research Institute, who made a dive to the clam site on 10 June, the colonies of *Calyptogena* sp. are circular, up to 60 cm in diameter and contain phenomenal densities of clams stacked on top of each other. One box core 250 cm<sup>2</sup> in cross-sectional area is said to have contained about 40 living clams, and during sampling some were crushed in the jaws of the box core releasing clouds of bright red blood. (The blood of vent organisms typically contains large amounts of haemoglobin.) On opening the box core on deck, a putrid smell was given off that made everyone feel sick, but hydrogen sulphide, which provides the "life-blood" of vent communities at spreading ridges, has not been found in anomalous amounts within the core and overlying waters, according to Dr Taira.

The colonies are aligned along north-east/south-west trending reverse faults associated with subduction and to a lesser extent along north/south strike-slip faults. The Tenryu Canyon runs along a major north/south transcurrent fault system which cuts into the mainland of Japan along the valley of the Tenryu River. Water inside the colonies shows positive thermal anomalies of a few tenths of a degree centigrade which may arise from connate water upwelling along the fault planes as the accretion prism of sediments flanking Japan is "squeezed" by subduction. The clam colonies thus in many rein-

the subduction zone off the Oregon/Washington coast, where it has been suggested that the vent organisms are powered by oxidation of methane contained in upwelling "old seawater" derived from underlying sediments (see *Nature* 313, 339; 1985).

Other organisms found in the vicinity of the clam colonies include squat lobsters, gastropods, several of sea anemone and polychaete worms, but no giant tube worms have yet been found. Dr Ohta hopes to present the preliminary biological findings at an international symposium on deep-sea biology to be held in Hamburg, West Germany, next week. The specimens have been divided equally between the French and Japanese.

## Soviet science

## Gorbachev calls for new reform

ACADEMIC institutes in the Soviet Union must be "turned around sharply" to concentrate on research which is "technological in its thrust", the Soviet Party leader, Mikhail Gorbachev, told the Central Committee last week. Among other things, he proposed the establishment, within the Soviet Academy of Sciences, of "integrated, inter-industry scientific and technical centres".

This, in effect, would reverse Khrushchev's reform of almost 25 years ago, when its academy was stripped of its applied science institutes. Since 1976, party plans and directives have increasingly called for the academy to play a greater role in "coordinating" the implementation of research. Industrial centres of the type proposed by Gorbachev have already been established; one example is the Paton Electric Welding Institute under the aegis of the Ukrainian Academy of Sciences. This experience, Gorbachev suggested, should now be applied at the all-union level.

This turnabout would not, however, mean a decline of interest in pure research. On the contrary, said Gorbachev, it must be given priority, with no quarter given to "weakness" or "sluggishness". The research potential of the universities, in particular, must be better deployed. According to "available assessments", Gorbachev said, higher education establishments could increase the volume of their research by between 100 and 150 per cent (presumably without additional resources). Gorbachev's emphasis on the academy and university sectors is due, it appears, to the poor performance of the research establishments of the various production ministries. The Soviet Union spends 5 per cent of the national income on science, almost 90 per cent of that in

Cameramen from the Japanese national broadcasting corporation, NHK, will now be allowed to make two dives on the *Nautille* to film the seafloor. Japan's biologists, who have not been included in the dive schedule despite predictions that "vent communities" might be found (see *Nature* 313; 1985), hope that NHK will choose to film the Tenryu Canyon clam colonies and perhaps collect some more specimens.

All this, however, depends upon *Nautille* being repaired in time to resume operations this summer. The electrical circuit of the submersible has been damaged and technicians and equipment must be flown out to Japan from France. At worst the Kaiko project may have to be delayed until next year, which could be a blessing in disguise as it would open a way for biologists to participate in the project. A decision on the future of Kaiko is expected this week. **David Swinbanks**

ministry institutes. More than half of all Soviet scientists work in them, and, according to Gorbachev, many of them, notably the Ministry of the Chemical Industry, have become "overgrown", with too many scientific institutes and pilot plants. Yet these are precisely the industries where there have been major shortcomings in developing new materials.

In his speech last week, Gorbachev complained that too many administrators, instead of finding a proper solution to a problem, shelve the issue by explaining that they are "carrying out an experiment", Gorbachev's answer is to strengthen the State Planning Committee, and to put into practice Lenin's dictum that the committee must be transformed into the country's "economic science organ, gathering together major scientists and leading specialists".

On the practical side, he urged that since the main weakness of Soviet industrial research lies in its isolation from production, many of the so-called "industrial" institutes should be immediately amalgamated with production enterprises. An immediate review should be carried out of how far the applied research institutes correspond to the current needs of industry.

Gorbachev also urged that something should be done about the declining prestige of engineers and the falling recruitment of talented young people into the technical sciences, which seems to have alarmed the Academy of Sciences. Gorbachev offers no immediate solution, but suggested that it may be time for a "serious reorganization" of higher education, meanwhile urging "measures to raise the social recognition" of engineers and technologists, including new pay deals.

**Vera Rich**



## Animal rights

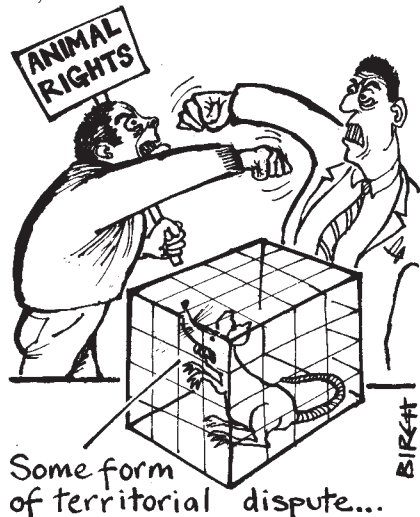
## Battle joined on animal lib

Los Angeles

CALIFORNIAN investigators whose research animals have been stolen, whose laboratories have been vandalized, whose work has been disrupted and whose very lives have been threatened by "animal liberation" activists, are fighting back.

Earlier this month, several of them testified before a Californian Assembly committee on public safety which subsequently approved a bill that would stiffen the penalties for stealing research animals. An animal "liberator" currently faces misdemeanour charges, punishable by up to three months in county gaol and a fine, provided the animal is worth less than \$400. The bill, if passed into law, would allow a judge to punish such a thief as a felon, with a 16-month to 3-year state prison term plus fines, no matter how inexpensive the animals.

"We are addressing the basic problem, which is interruption of research", says assemblyman William J. Filante, a northern Californian Republican who introduced the legislation. As the state legislature's only physician, Dr Filante said he feels an obligation to stiffen the penalties: "We are talking about vandalism, terrorism, destruction and theft".



Californian research facilities are threatened by ever bigger and bolder attacks, according to Sandra Bressler, executive director of the California Biomedical Research Foundation. The organization was set up last year, she says, to educate the public about how and why animals are used in biomedical research. California's leading universities, research clinics and other health organizations have joined the "fight back".

The problems began in 1982 when two monkeys used in brain research at the University of California (UC) at San Francisco were videotaped by interlopers. The tape was shown in Canada to raise money for animal rights groups. In 1983, two cats were stolen from a UC Berkeley

campus psychology laboratory and twelve dogs used in cardiovascular research were taken from the Harbor-UCLA Medical Center in Los Angeles. The dogs, each valued at \$5,000, wore pacemakers; research was delayed one year and the total loss was estimated to be £100,000.

In 1984, there were incidents of animal theft and vandalism at UC Sacramento and at the City of Hope Medical Center in Duarte. A \$400,000 cancer study at Duarte was set back three years. There were also cases of threats and paint-spraying at UC Davis and San Diego.

In January this year, 11 rabbits were taken from a Napa county animal shelter and the chairman of the department of surgery at UC San Diego received a death threat. Two months later, the home and car of the Los Angeles county animal control director were vandalized. And in April 467 animals were stolen from labor-

atories at UC Riverside, ruining eleven projects and causing damage assessed at \$683,000.

UC San Diego biology professor Mark Chappell lost 305 deer mice that he had painstakingly bred for five years to study variations in haemoglobin. Also in April, a student spirited away a cat being used to demonstrate premature infant resuscitation techniques at Stanford University.

None of the thieves, most of whom claim to belong to the Animal Liberation Front, has been caught, although there is one suspect from the Riverside incident and one person has been identified as passing on rabbits stolen from the City of Hope Medical Center.

The California legislature is under considerable pressure both from the biomedical community, which is powerful in California, and the animal rights activists, who are extremely vocal. Most legislators would like the problem to "go away", said an aide to Dr Filante. "But we expect it to stay a major issue for a long time. It'll be a big fight."

Sandra Blakeslee

## Plant biotechnology

## Young men go East to Georgia

Washington

THE University of Georgia, hoping to establish itself as a world-class centre for plant biotechnology, has acquired as a job lot a 20-strong research group from the University of Colorado. It has also committed \$5 million over the next five years to establish 25 new faculty positions in molecular biology. The state of Georgia has demonstrated its support with a promise of \$32 million for a new biotechnology building on the university's campus at Athens. The newly-hired research group is that of Peter Albersheim, who will be director of the new Center for Complex Carbohydrate Research. Albersheim let it be known last fall that he was planning to leave Colorado: Georgia was able to persuade him to Athens with an offer of almost 10,000 square feet of unused laboratory space in the Richard B. Russell Research Building, a facility of the US Department of Agriculture (USDA).

The new carbohydrate research group will work in collaboration with USDA scientists, and is expected to expand to a total staff of up to 40. Another reason Albersheim chose Georgia is the university's commitment to expand its biological research activities, which (including contract research) amounted to \$75 million last year. About half of the promised 25 new faculty positions are likely to be in Albersheim's group, which brings with it from Colorado \$1 million in research grants. Albersheim sees an impending golden age for the study of carbohydrates, which have tended to receive far less attention than other biopolymers. Now, he points out, it is becoming clear that carbohydrates, both individually and

combined with proteins, play an important role in controlling metabolism and may dominate immunological reactions. Genetic engineering companies that have hitherto concentrated attention on proteins will in future have to pay more attention to carbohydrates, Albersheim believes. The University of Georgia has long nursed aspirations to be renowned for something. When president Fred Davison started looking at the available options, plant biotechnology seemed a likely choice.

It is planned that the faculty expansion over the next few years will be complemented by an international Information Center for Plant and Animal Biotechnology. This scheme, still rather vague, is intended to provide information to plant and animal breeders who may be aware of the potential of the new biotechnology but who do not know where to find researchers working in relevant areas.

According to Leon Dure, a professor of biochemistry at Georgia, there is a "distinct gap" in the knowledge of plant breeders about how recent developments might help their work. Dure expects that the member institutes of the World Bank's Consultative Group for International Agricultural Research, which includes crop and animal breeding centres all over the world, will be among the users of the new centre. In its initial phase the centre will concentrate on putting researchers and potential clients into contact with one another; later, it is hoped that more technical information, including gene and protein sequences and possibly complete scientific papers, will be included.

Tim Beardsley



# Safety cabinets and AIDS

SIR — Many laboratory workers will be dismayed at the recent recommendation from the Advisory Committee on Dangerous Pathogens (ACDP) regarding the use of microbiological safety cabinets for the acquired immune deficiency syndrome (AIDS) agent human T-cell lymphotropic virus type III (HTLV-III). In this ACDP report<sup>1</sup> it is stated: "It is now clear that infection with HTLV-III may result in a fatal disease for which there is no effective prophylaxis or treatment. While there is still uncertainty about all the possible routes of transmission in the laboratory environment, care must be taken to avoid the dispersal of aerosols and for some work microbiological safety cabinets will be required. Because there are reservations about the level of operator protection which can be maintained by Class II cabinets under working conditions, for the time being, they must not be used for work with materials which contain the virus."

In many respects this conflicts with paragraph 5, Appendix A of the ACDP document *Categorisation of pathogens according to hazard and categories of containment* again published in 1984<sup>2</sup>, only a few months before the AIDS recommendations. This appendix states that "In certain conditions some Class II cabinets will provide a protection factor of the same order as Class I cabinets (that is, a factor of  $10^5$  or better. BS 5726: 1979<sup>3</sup>).

If it can be shown by in-use tests that this level is achieved consistently (as demonstrated by tests carried out at regular intervals, the frequency of which will depend on use) and provided that the local safety arrangements will allow, a Class II cabinet may be used for some work with group 3 pathogens where protection of the work is essential. . . ."

In the six years since BS 5726 was published, many laboratories have invested heavily in both Class I and II cabinets that meet the operator protection requirements of the standard. Cabinet manufacturers have responded well to these requirements with the result that many open-fronted cabinets can consistently provide more than the minimum protection factor of  $10^5$ . In addition, there has been a great improvement in the routine maintenance and certification of such cabinets. Many institutions now require certification to the filtration and containment requirements of the standard to be obtained twice per year for each cabinet.

The latest ACDP recommendations on the use of AIDS material in safety cabinets are questionable on two counts. In the first place they indict Class II cabinets in favour of Class I types and in so doing ignore the fact that Class I types are at least as easily disturbed by operator movements, by ventilation systems, by door movements and by people circulating within the laboratory. It is quite possi-

ble to reduce operator protection factors by one to two orders of magnitude by poor operator technique and lack of correct discipline.

Secondly, the recommendations totally disregard the current performance of many new Class II type cabinets which can produce consistent operator protection factors up to and beyond  $10^6$ . It has to be remembered that BS 5726 makes no distinction between the operator protection of Class I and II cabinets — both must have factors of at least  $10^5$ .

If there is so much uncertainty about the virulence of HTLV-III, then the very sensible procedures regarding the testing of operator protection factor initially outlined by ACDP for Class II cabinets<sup>2</sup> should be just as vigorously implemented for Class I types. As the weight of evidence does not favour transmission of HTLV by the airborne route, it is difficult to understand why Class I cabinets are allowed whilst similarly performing Class II cabinets are disallowed. Some of the work of this laboratory indicates that a number of the recently produced high performance Class II cabinets do, in fact, have higher nominal protection factors

and are more resistant to disturbances than many Class I cabinets. Much of this cabinet evaluation work has been published in the scientific literature<sup>5</sup> and has formed the basis for containment policy in the Medical Research Council. It is naive to think that tissue culture work will always escape contamination in Class I cabinets or that they are intrinsically safer than Class II types. The banning of Class II cabinets by ACDP for AIDS work is inconsistent with their other recommendations and will cause grave difficulties for laboratory workers who require both operator and product protection without the difficulties associated with Class III cabinets. On this aspect of containment the ACDP committee should think again.

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2. Advisory Committee on Dangerous Pathogens. *Categorisation of Pathogens According to Hazard and Categories of Containment* (Health and Safety Executive, London 1984).
3. BS 5726: *Specification for Microbiological Safety Cabinets* (British Standards Institution, London, 1979).
4. Clark, R. P. *Occupational Hygiene Monograph No. 9* (Science Reviews, Northwood, 1983); *Phil. Trans. R. Soc. Lond. B302*, 593-604 (1983).
5. Clark, R. P. & Hill, J. *J. clin. Path.* **34**, 12, 1404 (1981).

## Life and death

SIR — Rapid innovations in the biological sciences and technology are creating new and difficult ethical problems, as is evident to readers of *Nature* from numerous articles and letters. Most prominent are problems concerned with the early phase of the human life cycle. (See for example recent letters to *Nature* on the question "When life begins", *Nature* **314**, 492; 1985.) The "rights" of a human zygote, embryo and fetus, but for some reason not gametes, seem to evoke strong emotions.

New problems are also arising about when life ends. Problems of organ exchange necessitate a legal definition of the death of an individual.

At another level, what should be the status of human cells cultivated *in vitro*? These cells, in common with zygotes, have the potential, theoretical at the moment, to be regenerated if properly nurtured.

Further problems are now raised with the news of cloning of DNA extracted from an Egyptian mummy (*Nature* **314**, 576 and 644; 1985).

The unique genome of a human being apparently does not die when a person stops breathing or when electrical brain activity ceases.

The DNA molecules can be extracted and under proper conditions can be replicated. Thus by at least some definition of living matter the DNA is "alive". If the DNA is still alive, should we not change our accepted procedures of disposing of human corpses?

I suggest that the procedure of crema-

tion, sometimes associated with the saving of the ashes as a memorial, is improper. Instead it would make much more "biological sense" to save at least a sample of DNA from the deceased.

I envision "mausoleums" where sealed test tubes containing these DNA samples will be kept as an appropriate memorial to individuals. With the rapid progress of biotechnology, the potential for "resurrection" of at least some of one's traits is not remote.

AHARON GIBOR

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## Cows and goats

SIR — While Andrew Hill (*Nature* **315**, 222; 1985) shows deep understanding of the evolution of early African Hominoidae, his knowledge of Kalenjin nomenclature appears limited.

John Kimengich is surely John Kimnetich, which in Kalenjin means "born between 10 and 11 a.m. when the cows leave the cattle enclosure".

I am sure Dr Hill appreciates how in Kalenjin, as in English, omitting certain letters, for example the "o" from hominoid, can dramatically alter the sense.

MONIQUE BORGERHOFF MULDER  
(born between 9 and 10 a.m. when the  
goats go out, also known as Chemngeno)  
Tabarit village,  
PO Gorgor,  
Sotik,  
Kericho, Kenya

# Periodic extinctions undermined

*Last year's fashion for explaining a supposed 16-million-year periodicity in mass extinctions of species has been made to seem a little spurious. Defining a mass extinction is a more urgent need.*

THOSE who, last year, were enthusiastically speculating on the mechanism that might have been responsible for mass extinctions of living things at intervals of 26 million years will be given uncomfortable second thoughts by the article by Antoni Hoffman on p. 659 of this issue. For on the face of things, Hoffman has undermined the assumption on which all the excitement was based, the belief that there is a 26 million year periodicity to be explained. But human nature being what it is, it seems unlikely that the enthusiasts for catastrophism will now abandon their quest.

What Hoffman has done is to re-examine the palaeontological data on which rests the supposition that there is, or at least has been, a regularity in the timing of mass extinctions. The data consist of a catalogue of disappearances from the fossil record of families of marine animals compiled by J.J. Sepkoski and published, during 1982 and 1983, in the journal put out by the Milwaukee Public Museum. The first analysis of this material appeared in February 1984, in a paper by D.M. Raup and J.J. Sepkoski (*Proc. natn. Acad. Sci., U.S.A.* **81**, 801-5; 1984). It advanced the conclusion that mass extinctions of marine animals have taken place at times in the geological past separated by intervals of 26 million years or an integral multiple thereof.

That Sepkoski's original data may not be robust enough to support this dramatic conclusion has always been a possibility, as Raup and Sepkoski themselves acknowledged. The most obvious difficulty is the unavoidable uncertainty in assigning absolute ages to the successive geological stages recognizable only by the fossil assemblages that define them. For the past 100 million years, including the Cretaceous/Tertiary transition at 65 million years, the dating of the stages is reasonably good, with the result that most geological chronologies are consistent during that period, but disagreement is rife for earlier times. Raup and Sepkoski gave reasons, in their paper, for adopting the chronology due to W.B. Harland; the first column (labelled **a**) in Hoffman's Fig. 1 shows how the times of mass extinctions would have been changed if they had followed another equally plausible chronology.

The nub of Hoffman's argument is, however, more subtle. Because palaeontological stages are defined by their fossil

assemblages, each must differ from its predecessor and its successor by the appearance or the extinction of at least one family. So, Hoffman argues, distinguishing between a normal stage and one that qualifies as one of mass extinction requires the consistent application of some objective criterion. He would prefer a criterion based on the rate or probability of extinction per unit time (say a million years) rather than one based on counting the number of family extinctions in a stage, which he says is biased towards periodicity.

So much is evident in the work of Raup and Sepkoski, where it is a necessary condition for associating a particular stage with a mass extinction that the number of families extinguished should be greater than in the preceding and succeeding stages. The effect of that is to exclude the possibility that consecutive stages could be associated with mass extinction, which is bound to prejudice the analysis towards a periodic interpretation. Indeed, Hoffman argues, if the probabilities of extinction in successive stages are entirely independent, simply looking for peaks in the record of extinctions implies a one-in-four chance that any stage will be recognized as a time of mass extinction and, given the exclusion of the possibility that consecutive stages may be thus described, the analysis is certain to yield the conclusion that, on the average, extinction peaks occur every four stages. This, says Hoffman, is the origin of Raup and Sepkoski's periodicity.

The chief effect of this argument will be to emphasize how tricky is the concept of mass extinction. To be fair, many of the pitfalls were acknowledged by Raup and Sepkoski. The plain truth is that the disappearance of one or several families of genera from the fossil record cannot be counted as proof of a mass extinction, in the ordinary meaning of that term, without much more detailed scrutiny of the data than mere enumeration. How abrupt was the transition? Did all species disappear simultaneously, or can something be learned of the nature of the extinction from the sequence in which they disappeared? And since there are many possible causes of the extinction of species, genera and even whole families, ranging from evolutionary competition and climate change to external influences such as are involved in cometary or other impact, what can be said about the mechanism?

The need to give at least partial answers to these questions should now be plain.

Where does this cautionary tale leave the explanations constructed to account for periodic extinctions, particularly for that which seems to have carried off the dinosaurs at the Cretaceous/Tertiary boundary? One conclusion that stands out from Hoffman's argument is what he calls the Maestrichtian stage at the end of the Cretaceous was, by all the different criteria applied, an extinction period.

Thanks to the work of the Alvarezes, father and son, and their associates, this is also recognizable by the world-wide presence of material rich in iridium at the physical boundary between the Cretaceous and the Tertiary. The most compelling evidence that this material came from the impact of some extraterrestrial object with the Earth is the finding of cracks caused by explosion stresses in a high-pressure phase of quartz associated with the iridium layer (B. F. Bohor *et al. Science* **224**, 867-8; 1984). The case for this impact is strong (and would be even stronger if there were some trace of where it may have happened), but cautious people will insist that there is as yet only circumstantial evidence that the impact caused the extinction.

By contrast, the explanations of periodicity in the pattern of extinctions assume a somewhat academic character, which is not the same as saying they are uninteresting. The most plausible source of material hitting the surface of the Earth is the distant ring of comets called the Oort cloud, from which material may be ejected towards the inner Solar System either by the perturbations of an unseen companion of the Sun (called Nemesis) or by perturbations of the Solar System caused by its periodic passage through the plane of the Galaxy.

Last year's rush of explanation has indeed thrown light on questions such as the stability of the Oort cloud, and of distant (and hypothetical) elements of the Solar System. But the case for Nemesis has now wilted. And those who are attached to the notion that extinctions are caused by the oscillations of the Solar System above and below the plane of the Galaxy, among whom Clube and Napier (see *Nature* **283**, 455; 1979) seem to be the first, might now usefully turn the problem around, and ask what happens to the Solar System on these excursions through the galactic plane.

**John Maddox**



## Transgenic animals

# New advances in the field

from Robin H. Lovell-Badge

INJECTION of new genetic material into fertilized mouse eggs followed by development of the eggs into transgenic mice in foster mothers has become an almost routine tool in the analysis of a wide range of biological problems<sup>1</sup>. If the same technique could be used for farm animals it could be of great practical and economic importance, allowing the transfer of characteristics from one breed to another much more rapidly than by conventional breeding. More dramatically, it could allow genetic transfer between species. The paper by Hammer *et al.*<sup>2</sup> on page 608 of this issue is the first demonstration that it is indeed possible to use microinjection to obtain integration into the genome, and in some cases expression, of exogenous DNA in large mammals—namely rabbits, pigs and sheep.

To work with large domestic animals rather than mice requires a considerable investment, particularly in the number of animals required. While it is normal to obtain up to 40 eggs from a single super-ovulated mouse, a ewe gives on average only three or four. Reimplantation of manipulated embryos may pose an even greater problem. Even though most will not survive, mice can tolerate up to 20 offspring, whereas sheep and cattle will not give birth to more than two. The occurrence of freemartins would be an additional problem with cattle.

These considerations have presumably influenced the selection of species used by Hammer *et al.* Pigs offer advantages because they are multiparous, sheep are an example of a difficult species and rabbits, while a reasonably convenient laboratory animal (and many a farmer's nightmare), are similar to pigs and sheep in many aspects of their early embryonic development. It is also essential to choose techniques that are efficient. It is clear from microinjection of tissue culture cells<sup>3</sup> and mouse embryos that transformation frequency is very low unless DNA is injected into the nucleus. But the eggs of many domestic animals have such an opaque cytoplasm that it is impossible to see their pronuclei or nuclei with conventional light microscopy. DNA-specific fluorochromes have been used to visualize nuclei in early bovine embryos<sup>4</sup>, but the damage induced by ultraviolet light can severely compromise their development.

For sheep embryos, Hammer and colleagues have solved the problem to a large extent (but see below) simply by the use of interference contrast microscopy. More drastic procedures were necessary for the pig<sup>5</sup>. Centrifugation of the embryos at 15,000 g for three minutes separates the

cytoplasm into three layers. Most droplets and granules, corresponding to the lowest buoyant density but greatest optical density, are found at one pole. The equatorial region contains a band of small clear droplets and the remaining half of the zygote has a translucent appearance. Nuclear structures may be found in either of these regions and are therefore clearly visible. The centrifugation of the pig embryos seems not to affect their subsequent development. It may also be a useful technique for other species, notably cattle, to allow experiments involving nuclear transfer for investigating the possibility of cloning<sup>6</sup>, or parthenogenic development<sup>7</sup>, of farm animals.

The gene chosen by Hammer *et al.* for injection into the nuclei of embryos of all three species is the mouse metallothionein-human growth hormone fusion gene that has been used in the same laboratory (Ralph Brinster's) for experiments with mice<sup>8,9</sup>. In each case about ten per cent of the injected embryos survived to term after reimplantation into foster mothers. The frequencies of fusion gene

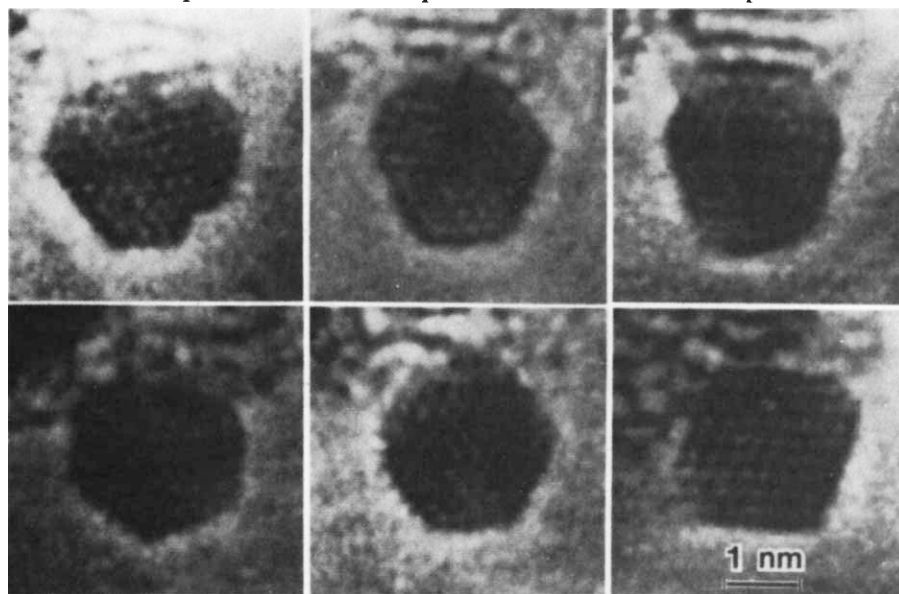
integration in the rabbit (12.8%) and pig (11.0%), although lower than the overall frequencies in mice (about 27%), are good, and other workers using these species will be much encouraged.

By contrast, the results with sheep are disappointing. Only one out of the 73 fetuses or newborn carried the injected fusion gene and even then it was obviously deleted or rearranged. It is conceivable that sheep embryos lack the appropriate mechanisms that fortuitously integrate exogenous DNA, but it seems more likely that some aspect of the technique needs refining. Perhaps more copies need to be injected and perhaps the visualization of the pronuclei needs to be improved (nuclear structures were not seen in a proportion of fertilized sheep eggs and these may correspond to more 'receptive' stages). Otherwise it may be necessary to adopt alternative strategies such as the use of retroviral gene vectors that are just beginning to work with mice.

Ovulation and fertilization are not very synchronous in the rabbit, pig and sheep, so it was necessary for Hammer *et al.* to inject embryos that ranged from 1-cell to 4-cell stages. For mice, even when the injections were at the pronuclear 1-cell stage, some of the transgenic animals were mosaic, presumably because gene integration was delayed. When this mosaicism was in the germline, few offspring inherited the introduced gene sequence<sup>10</sup>.

## Japanese gold in atomic motion

Use of a high-resolution electron microscope and video recorder has allowed the first real-time recordings of the motion of individual atoms. The pictures above show an ultra-fine particle of gold (18 Å across and containing about 500 atoms) photographed using the new technique by Sumio Iijima, a group leader in Japan's ERATO ultra-fine particles project (see *Nature* 305, 373; 1983). It can be seen that the shape of the particle changes over time, in discrete steps from top left to bottom right with all the gold atoms making up the ultra-fine particle moving cooperatively to form various crystalline structures. The gold atoms in ultra-fine particles thus seem to behave in a 'quasi-solid' manner with some properties like those of a liquid—one gold particle can even 'swallow-up' a smaller one. These phenomena await theoretical explanation.



It will be interesting to see if the same result emerges from breeding studies of the transgenic pigs and rabbits.

Expression of the fusion gene was observed in some of the transgenic rabbits and pigs, with a variation between individuals similar to that in mice and presumably due, at least in part, to different integration sites<sup>8,9</sup>. Further work will be necessary to show that the mouse metallothionein promoter works 'correctly' in rabbits and pigs, although it seems likely that the rules governing expression of exogenous genes in mice will be generally applicable to other mammals. Let us hope so, because it would be useful to derive transgenic mice to test the suitability of particular gene constructs before introducing them into farm animals.

It has long seemed likely that it would be possible to introduce genes into farm animals, and many groups around the world, often with considerable investment behind them, are attempting to do so. What are the potential gains? Some of the most obvious are improved efficiencies in reproductive performances and weight gain, increased disease resistance and changes in wool texture. The introduction of extra growth hormone genes would be one way to increase growth but, although the results with mice have been dramatic<sup>8</sup>, there has been no effect so far on pigs or rabbits<sup>2</sup>. It may be possible to produce transgenic animals with an increased rate of ovulation by, for example, increasing their gonadotrophin or lowering their oestrogen levels<sup>11</sup>. (Impaired fertility was an unexpected characteristic of some of the growth-hormone transgenic mice<sup>12</sup>.) It may also be possible to increase ovulation rate with specific genes such as the *Booroola* gene of sheep; the action of this gene is not entirely clear, but it may

increase the sensitivity of granulosa cells to follicle stimulating hormone so that follicles mature when smaller<sup>13</sup>. The association of specific histocompatibility antigens with particular diseases suggests that it may be possible to introduce non-associated alleles to induce resistance. Another possibility is the introduction of keratin genes to alter the properties of wool or hide.

Finally transgenic farm animals could be used to carry novel gene constructs as production systems for biologically and clinically important peptides and proteins. This should be particularly useful where expression in mammalian cells is required to give appropriate post-translational modification, or where the gene products are difficult to purify in other expression systems. It should, for example, be possible to use appropriate promoters to ensure that the introduced genes are expressed in the mammary glands of sheep or cattle, so that the gene products can be isolated from milk. Lest anyone should think this idea as silly as is that of converting the forelimbs of sheep into needles so they can knit, they should be aware that the use of silkworms for the production of interferon has just been reported<sup>14</sup>. □

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## Protein folding

# ... yet there is method in't

from Roger Pain

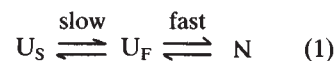
THE mechanism of protein folding has developed from a purely fundamental study into one which is also crucial to the recovery of proteins made by biotechnological means in microorganisms. While the patent literature shows that some degree of success can be achieved by empirical approaches, further improvements in the economy, scale and efficiency of protein recovery can be expected once the refolding pathway is better defined. Since the rate at which folding occurs *in vivo* provides one of the major constraints on any theory, *in vitro* kinetics have been studied intensively. Much of this work has been centred on ribonuclease (RNase) and its progress has been followed in these columns<sup>1-3</sup>. But now there are some new twists to the tail.

It is because its amino-terminal surface

helix should constitute a revealing model for formation and association of a well defined element of secondary structure with a larger protein fragment that RNase has received so much attention. An added advantage is that one can compare RNase A, which is a single polypeptide chain, with RNase S, in which the helix, or "S-peptide", is associated with the remainder, or "S-protein", only by non-covalent forces following cleavage of the chain between residues 20 and 21.

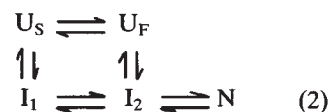
Not surprisingly the observed kinetics of unfolding and refolding are frequently biphasic or more complex but, confusingly, such kinetics cannot be assigned to conformation changes alone. Brandts and colleagues have shown that the existence of at least two unfolded states, which contain different proline isomers and are

indistinguishable by accessible spectroscopic techniques, would lead to multiphasic kinetics, even if the actual folding process were simple and monophasic<sup>4</sup>. In the absence of conformational constraints, proline distributes itself between the *cis* and *trans* isomers with half times of a few minutes; folding to a conformation requiring a unique isomer will result in biphasic kinetics from fast and slow refolding forms:



where  $U_S$  and  $U_F$  are unfolded forms, and  $N$  is the native protein.

The characteristic feature of this theory is that proline isomerization occurs in the unfolded state. Subsequent experiments, initially from Baldwin's group, were interpreted as showing that proline isomerization can occur in a collapsed, partially-folded state  $I$  according to the scheme:



where the intermediate form  $I_1$  contains incorrect proline isomers and  $I_2$  contains native proline isomers. In this sequential model, the slow phase of the kinetics arises from the existence of conformational intermediates as well as from proline isomerization<sup>5</sup>. It is further claimed by Baldwin's group<sup>6</sup> that isomerization can be speeded up in the condensed phase ( $I_1 \rightleftharpoons I_2$ ). Schmid has suggested that an enzymically-active folded state, which he showed to be an intermediate on the folding pathway, contained an incorrect proline isomer<sup>7</sup>.

Part of the difficulty in resolving these opposing views lies in the lack of methods which enable each intermediate to be fully characterized and each kinetic phase to be assigned to a particular molecular transition. While, for example, NMR has been used to follow the *cis-trans* isomerization in small peptides, it has not yet been successful in proteins. In 1983, Brandts (with Lin) returned to the disputation with an elegant method for following the kinetics of proline isomerization: cleavage by certain proteinases of the bond between proline and its neighbour in a polypeptide is dependent upon the isomeric state of the proline, allowing the proportion of *cis* and *trans* isomers to be estimated as a function of time for a particular proline residue<sup>8</sup>. Lin and Brandts show that proline 93 is 100 per cent *cis* in native RNase A but only 70 per cent *cis* in the reversibly unfolded protein. Under refolding conditions, the relaxation time for isomerization from 70 per cent to 100 per cent *cis* is 90 s at 10°C. Parallel measurements of regions of ribonuclease activity showed that 30 per cent of the activity is recovered in a slow phase with a relaxation time of



100 s. The agreement of amplitude and rate shows that the slow phase in the recovery of activity is rate limited by the isomerization of proline 93, and argues for refolding taking place only from chains in which that proline is in the native *cis* form.

Lin and Brandts have now studied two more of the four proline residues in ribonuclease. Proline 117 is most probably completely *trans* in both native and unfolded states and therefore will make no contribution towards kinetic complexity. On the other hand proline 114 is completely *cis* in the native protein and 50 per cent *cis* in the reversibly unfolded state, which correlates with the low-amplitude slowest refolding phase. This residue must be in the *cis* configuration for the protein to regain activity<sup>9</sup>.

The major fastest phase of the refolding kinetics is thus unaccounted for and the isomerization of proline 42 has yet to be investigated — the experiments will be slow and tricky. While Lin and Brandts are willing to acknowledge the existence of the non-native active intermediate their view is that, if it accounts for the major refolding phase, it is unlikely to involve proline isomerization. In support of this, they quote unpublished work of Fisher *et al.* This group has purified a new enzyme, peptidylprolyl-*cis*, *trans*-isomerase<sup>10</sup>, which catalyses the kinetic steps associated (by Lin and Brandts) with isomerization of prolines 93 and 114 but has no effect on the major fast phase. Does this enzyme require the ribonuclease to be unfolded in order to catalyse the isomeri-

zation of proline, offering support for the Brandts model? To add to the confusion, Schmid, Buonocore and Baldwin<sup>11</sup> have not only further confirmed their sequential model but have also strengthened the evidence that the major phase shows the characteristics of proline isomerization.

On a slightly different tack, a further advance in assigning kinetic phases to distinct regions of the ribonuclease structure has come with the discovery by Labhardt that the S-peptide and S-protein fragments of RNase S can be distinguished by circular dichroism<sup>12</sup>. From the kinetics of conformational changes of both the S-peptide and the S-protein during their association and refolding reactions, Labhardt comes out firmly in support of a sequential type of folding pathway. Intriguingly, his experiments show that the S-protein folds (to a form as yet uncharacterized) in a fast phase before the S-peptide folds to its native helical structure. This fast phase corresponds to 65 per cent of the total change, greater than the proportion of fast folding species  $U_F$  in scheme 2. The folded form must correspond in part at least to  $I_1$ .

Since Labhardt has already shown that recombination between S-peptide and S-protein occurs fast and very early in the folding pathway of RNase S<sup>13</sup>, we have to envisage, contrary to most previous expectations, that the association between these two regions occurs at a stage when the S-peptide, and possibly the S-protein, possess no native-like conformation. This would be in keeping with the poorly hydrogen-bonded associated species detected by NMR<sup>14</sup>. If Labhardt's results, obtained with a relatively 'simple' system, are of general application, the model of 'flickering clusters' of secondary and super-secondary structure, which associate to be stabilized in the native conformation, will have to be considerably revised.

The circular dichroism results have also raised a problem relating to the unfolding

kinetics. The unfolding rates for RNase S and S-protein, which have been followed by monitoring changes specifically in the S-protein moiety, parallel the formation of the slowly refolding species  $U_S$  which contains non-native proline isomers (scheme 2). The random isomerization of proline to form  $U_S$  had previously been thought to occur in the spectroscopically 'silent' reaction ( $U_F \rightleftharpoons U_S$ ) but Labhardt's results suggests that, in terms of the model of scheme 2, there must be a rate-limiting proline isomerization  $I_2 \rightarrow I_1'$  which is faster than  $I_2 \rightarrow U_F'$ , followed by a rapid conformational unfolding  $I_2 \rightarrow U_S$ . Alternatively, the rate determining step is a conformational unfolding  $I_2 \rightarrow U_F'$  followed by a rapid isomerization to  $U_S$ . Neither alternative fits easily with the expected relative rates of these processes.

The frustrating aspect of the whole question of proline isomerization is that, however the issue is resolved, it will tell us nothing about the actual *mechanism* of protein folding. Though it is becoming possible to see some of the detailed processes at work in the folding of ribonuclease, the methods have not yet overcome the kinetic madness of the system. □

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### 100 years ago

AN excellent opportunity of observing the aerial means of propulsion in the flying fish was afforded me during a six days' calm lately when crossing the Bay of Bengal. This must be my excuse for again touching this subject. I watched day by day some hundreds rise under the bows of the ship. The water surface was a glassy calm. As each fish rose it spread its wings at once, apparently beating the surface with them two or three strokes before they steadied out. The tail which, of course, under water was in rapid motion, to escape from the ship, now gave ten or a dozen rapid beats, which could be counted by the ripples on the still surface, and the fish was off in aerial flight. As each fish lost the impetus of the first rise, which generally happened at about forty yards, the binoculars showed us the anal fins, which had till now been fully extended, drooping to feel the water. As soon as the surface was felt the tail was quickly introduced, and five or six smart strokes, also indicated by ripples, brought the impetus up again and carried the fish about another thirty yards, when another droop sent it on again, and so forth, some of the older fish travelling in this way 400 to 500 yards.

From *Nature* **32** 147, 18 June 1885.

### Astronomical spectroscopy

## Interstellar space contains the largest encountered atoms

from William D. Watson

OBSERVATIONS published on page 647 of this issue<sup>1</sup>, in conjunction with recent Soviet publications<sup>2,3</sup> highlight the presence of extremely high Rydberg states — in which the outer electron of an atom has been excited to very high energies — in the interstellar medium. These results confirm and significantly extend recent identifications<sup>4,5</sup> of spectral lines at radio frequencies an order of magnitude lower than those at which lines had previously been detected. Discoveries of new lines at radio wavelengths have until now tended

to be associated with the high-frequency (millimetre and submillimetre) end of the spectrum.

Because of the appearance of several lines in the low-frequency series, it is clear that they result from radiative transitions involving extremely high Rydberg states (or radio-recombination lines) and changes of one unit in the principal quantum number of the state (alpha transitions). The lowest frequency at which these lines have been detected corresponds to the 732 alpha transition, which

indicates that the largest atoms that have yet been directly encountered in any environment are present (the radius of the Bohr orbit for a principal quantum number of 732 is 0.003 cm). These spectral lines are almost certainly due to atomic carbon.

There is evidence of pressure broadening in the new observational data. This phenomenon has not been detected previously at any wavelength in spectral lines associated with the interstellar gas clouds. Taken together for the first time, pressure broadening and the change from emission to absorption which is evident in recombination lines provide new diagnostic methods to delineate physical conditions of interstellar gas clouds. These clouds provide information about the process of star formation.

Radiofrequency transitions of atoms between adjacent or nearby Rydberg states with large principal quantum numbers have commonly been observed in astronomy since their first detection in the 1960s. Studies of these radio-recombination lines have had an impact on several astronomical issues<sup>6</sup> including, for example, the abundance of helium (which has cosmological importance) and the physical state of the star-forming gas (molecular cloud). They are observed primarily in the diffuse gas of our Galaxy, in either the hot, ionized gas of H II regions where lines of hydrogen and helium are seen or in the mostly neutral, cold gas of molecular clouds where the lines of carbon and sulphur are found. Before the prediction in 1959 by N. S. Kardashev that radio-recombination lines should be detectable, it was not appreciated that transitions between states with very high principal quantum numbers could be observed without overlap because of line broadening. High Rydberg states of atoms are also of interest to atomic physicists (see, for example, ref. 7).

Until recently, the principal quantum numbers of the radio-recombination lines ranged from about 40 to 300. As confirmed by the new observations, the range of principal quantum numbers that can be involved and the characteristics of observable transitions have been dramatically expanded. The current data substantiate the earlier proposal<sup>4</sup> that spectral features detected at frequencies near 26 MHz in the direction of Cassiopeia A by A. A. Konovalenko and L. G. Sodin<sup>5</sup> should be identified as radio-recombination lines (alpha-transitions) involving the principal quantum numbers 630–640 of neutral atoms of an element other than hydrogen or helium. Except for these two elements, the isotope shifts are not large enough compared with the breadth of the lines to make a unique identification based on frequency alone. General considerations<sup>4,8</sup>, as well as the association of these features with concentrations of neutral gas, strongly suggest that the atoms in question are carbon atoms. Carbon is the most abun-

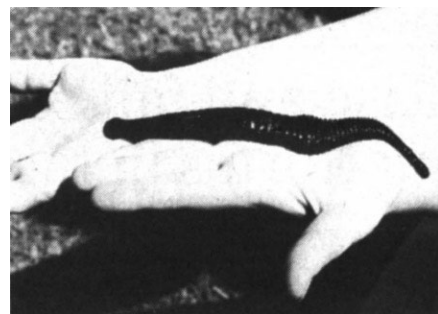
dant element for which the ionization potential is lower than that of atomic hydrogen. Thus, it can occur primarily as C<sup>+</sup> because of ionization by starlight in regions where hydrogen is in neutral form. The high Rydberg states involved in radio-recombination lines are populated primarily by the recombination of free electrons with the ions.

The recent observational studies<sup>1–3</sup> report the detection of the series of recombination lines far beyond the 26 MHz region described previously. In the direction of the radio source Cassiopeia A, the series is found to continue down to 16.7 MHz (732 $\alpha$ ) and up to 68 MHz (456 $\alpha$ ). Analogous, but more limited, sequences of such radio-recombination lines have now also been detected in several other directions in the Galaxy.

The idea of such 'large' atoms is, in itself, fascinating. As a result of the very weak binding of these Rydberg electrons, they interact strongly with their environment — that is other particles and radiation. One consequence is the observation of line breadths which exceed that due to Doppler broadening, the broadening presumably resulting from collisions with free electrons.

With increasing principal quantum number, the rate for collisionally induced transitions increases rapidly, whereas the loss rate from the Rydberg states due to spontaneous, radiative decays decreases at a comparable rate. The net effect is a greatly enhanced tendency for the excitation temperature of the very low-frequency recombination lines to approach the kinetic temperature of the gas. As a result, this new class of recombination lines has only been detected as absorption features in the spectrum of background, continuum radio emission. In contrast, at the lower principal quantum numbers ( $\leq 300$ ) the populations of the states are expected to be inverted. Before the recent studies involving principal quantum numbers greater than about 450, radio-recombination lines had, in fact, always been detected as emission features. Thus, there is a change from emission to absorption that occurs with increasing principal quantum number. For alpha-transitions and representative physical conditions, the predicted transition tends to occur near 400 (ref. 8). The critical principal quantum numbers at which the change occurs do depend upon the physical conditions; hence, additional information for delineating the physical conditions of interstellar gas clouds can be obtained.

Although the first detection of pressure broadening and the change from emission to absorption by recombination lines are the most striking features of this new class of spectral lines, more detailed theoretical and observational studies may elucidate additional effects of diagnostic value for interstellar gas clouds. The relative importance of various physical effects associated with Rydberg atoms tends to increase



The giant Amazon leech, *Haementeria ghilianii*, which grows up to 0.5 metres in length. Its salivary gland secretes the specific fibrinolytic enzyme hementin and other biologically active substances of potential scientific and medical use. The gland provides a unique preparation for studying excitation-secretion coupling in single cells, because the cells are very large and are not electrically coupled; see the paper by P. G. Jones, R. T. Sawyer and M. S. Berry on page 679 of this issue.

rapidly with increasing principal quantum number. For example, pressure broadening by the free electrons increases approximately as the fifth power of the principal quantum number in certain relevant regimes. There is also evidence<sup>2,3</sup> that the strength of the lines is influenced by a resonance in the recombination<sup>8,9</sup> which is related to the fine-structure excitation of C<sup>+</sup>. Rough assessments<sup>4</sup> of the expected strengths suggest that these recombination lines are likely to be widely observable in the gas of the Galaxy — a prediction tentatively supported by detections in several directions in the Galaxy other than towards Cassiopeia A. In the absence of a conveniently located compact source of continuum radiation, the diffuse synchrotron emission of the Galaxy is bright enough at the very low frequencies to provide an adequate continuum source against which the absorption can be observed. Finally, the occurrence of spectral lines at these very low frequencies provides a new opportunity for radiotelescope observers and investigators with an orientation towards very low frequencies. □

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## Ocean Drilling Program

# Rise and fall of carbonate platforms in the Bahamas

from the Leg 101 shipboard scientific party\*

LEG 101, the first international voyage of the Ocean Drilling Project, began on 31 January, 1985 in Miami and ended there on 14 March after 11 sites had been drilled in the Bahamas archipelago. The decision to begin this new phase of deep-sea drilling with a study of carbonate platforms reflects the growing interest of earth scientists in the anatomy of large sedimentary bodies.

The drilling revolved around two themes: the origin of the present array of platforms and basins, and the study of platform flanks, their facies patterns and their response to sea-level fluctuations that entail flooding and exposure of the banks. The origin of Bahamian banks and basins continues to be the subject of intensive debate because the pattern is unusual for passive ocean margins. In the past decade, the discussion has been governed by two fundamentally different concepts. One of them, the graben hypothesis, calls for strong fault control of the bank-and-basin pattern. It assumes that the present deep-water basins originated as grabens in the rift stage of the Atlantic and have remained topographic lows ever since<sup>1</sup>. On the other hand, the megabank hypothesis<sup>2</sup> assumes that rift structures and other inherited tectonic features were soon smothered by sediments during the spreading phase of the ocean and that for most of the Jurassic and the early Cretaceous, Florida, together with the north-western Bahamas and northern Cuba, formed a single, flat-topped carbonate platform – the 'megabank'. In the Cretaceous, this platform gave way to the pre-

sent array of banks and basins.

Debate continues in the wake of drilling. Most of us feel that Leg 101 and the preceding seismic-site surveys have greatly strengthened the megabank hypothesis. However, at least one of us (Mullins) considers the evidence inconclusive, mainly because we failed to reach the relevant seismic horizon in the Straits of Florida and Northeast Providence Channel, and prefers to emphasize that deepwater early Cretaceous sediments were recovered on Leg 77 of the Deep Sea Drilling Project, in dredge hauls from Samana<sup>3</sup>, and also on this leg from site 635. On the basis of this evidence multiple bathyal environments may already have been established at that time.

Leg 101 recovered shallow-water carbonates and evaporites of the megabank under the southern Blake Plateau. In the Straits of Florida, the carbonate platform was not reached but a close stratigraphic tie was established with an exploration well on Great Bahama Bank, 60 km to the north-east. The continuity of seismic records between site 626 and the Great-Isaac 1 well strongly suggest that the entire Straits of Florida is underlain by a continuous carbonate platform. Furthermore, the two holes indicate that rather than remaining stationary since the Mid-Cretaceous, the margin of Great Bahamas Bank has alternately retreated and prograded tens of kilometres during the past 100 Myr. Blake Plateau and Little Bahama Bank display a similar rhythm of mid-Cretaceous drowning and retreat followed by Late Tertiary expansion of the

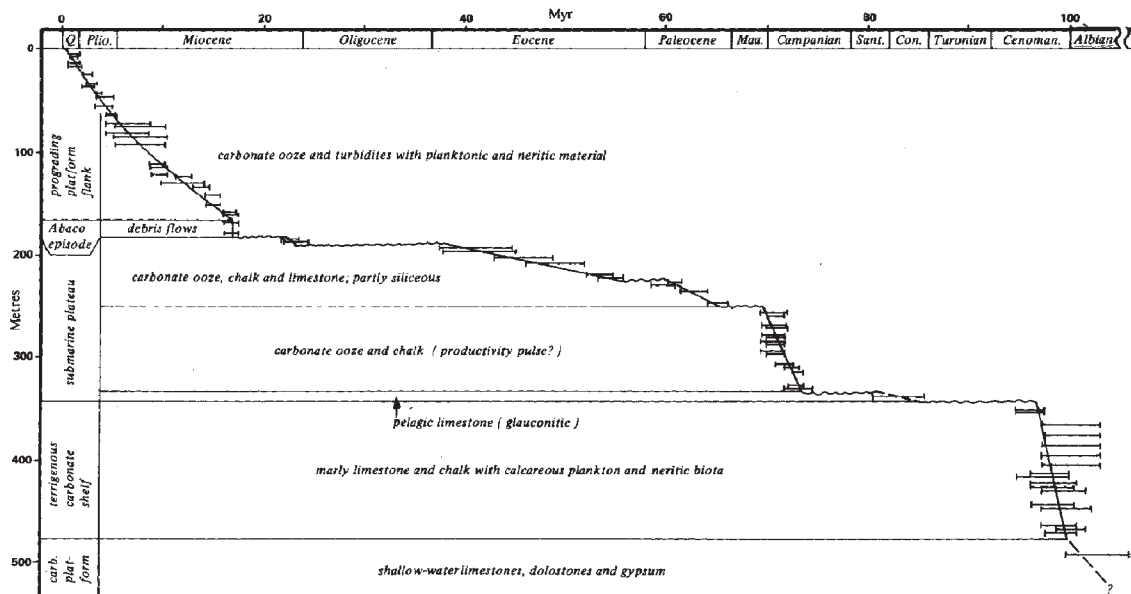
platforms (see figure). The evidence for wide horizontal migration of the platform margins and for shallow water carbonates underneath some Bahamian basins is not easily reconciled with the graben hypothesis of the Bahamian troughs<sup>2</sup>.

Mid-Cretaceous disintegration of the megabank does not seem to be related to local tectonic deformation but rather to a crisis in the depositional system. The drowning of the Bahama platform is part of a world-wide extinction of carbonate platforms that coincides with a tendency towards oxygen deficiency in the oceans<sup>4</sup>. Exploring links between anoxia and platform drowning remains one of the most challenging tasks for the shipboard party.

While for most of us the drilling tipped the balance in favour of the megabank-hypothesis, some modifications of that concept are called for. The stratigraphy of the Leg 101 sites, along with recent dredging results, indicates that mid-Cretaceous platform drowning was not synchronous throughout the archipelago and that it predates the mid-Cenomanian sea-level cycle of Vail *et al.*<sup>5</sup>. Drowning seems to have progressed in steps from the south eastern perimeter of the megabank to the centre. The oldest known deep-water sediments at the southeastern tip of Great Bahama Bank (Samana) are Neocomian in age<sup>3</sup>; 350 km farther north-west, at site 635, they are Albian; at site 627 and in the Great-Isaac 1 well, in the centre of the archipelago, they are Cenomanian in age.

Platform flanks were studied in two three-hole transects with the hydraulic piston corer. The response of platform flanks to sea-level fluctuations has recently become the focus of a lively discussion<sup>6-8</sup> and was one of the principal study objectives of Leg 101. Vail *et al.*<sup>5</sup> assume that sediment is fractionated between shelf and basin in only one way during a sea-level cycle: during high stands of sea level most sediment is trapped on the inner shelf; during low

Age versus depth profile and geological history of site 627 on the southern Blake Plateau. Drowning of the carbonate platform during the mid-Cretaceous crisis is followed by partly terrigenous deposition on a shelf, and by 80 Myr of slow pelagic deposition on a submarine plateau. In the past 20 Myr, the area has been influenced by the prograding Bahama platform again. "Abaco episode" is a wide spread incidence of debris flow deposition in the middle Miocene documented in the Blake Bahama-Basin DSLP Leg 44 (ref 9).



stands, when rivers carry their load right to the shelf edge, most material escapes to the deep sea. Results of Leg 101, along with a growing number of data on the late Pleistocene indicate that carbonate platforms do not fit this trend.

Preliminary comparisons of stratigraphy on the Bahama banks and in the basins strongly suggest that periods of low sea level (and bank exposure) 3 Myr BP and 5–6 Myr BP correspond to intervals of slow deposition and scarcity of turbidites in the basins. When sea level was high and the banks were flooded, sedimentation rates in the basins increased dramatically. This observation supports the concept of 'high-stand shedding' of platforms as opposed to the generally accepted phenomenon of 'low-stand shedding' of terrigenous continental margins.

The fine sediment exported by the platforms consists largely of aragonite, a form of calcium carbonate that is metastable in most burial environments and is rarely found in pre-Quaternary pelagic sediments. On the flanks of the platforms, bank-derived aragonite mixes with the remains of calcitic plankton. Leg 101 drilling provided the first glimpse of the burial history of this peculiar type of carbonate ooze. The first-order trend shows a down-hole decrease of aragonite and its replacement by calcite cement. Whereas calcite cementation is more rapid than in pelagic carbonate ooze, aragonite dissolution was found to be unexpectedly slow: 7 Myr-old limestones were found to contain 15–20 per cent aragonite. Superimposed on this first-order trend are cyclic variations in aragonite content. In the Quaternary, these have been shown to reflect climatic cycles. Further study of the Leg 101 cores is expected to reveal the Neogene climatic history of the platforms.

Hydrocarbons were encountered at several sites and led to abandonment of two holes. Oil stains and wet gas were

found in Cretaceous dolomites on southern Blake Plateau, and tar impregnations appeared in Miocene chalks in the Straits of Florida and in turbidites in Exuma Sound. All these are interpreted as hydrocarbons that have migrated from deeper parts of the section. In accordance with ODP practice, these holes were plugged with cement.

Leg 101 has added a dimension of Earth history to the numerous studies on modern platform flanks. The modern sediments of the Bahamas, often used as a standard for the interpretation of the geological record, have provided the key to their own past. This part is more varied and turbulent than previously believed. Periods of crises and of rapid expansion figure prominently besides intervals of steady growth. These changes seem to be governed by a complex interplay of oceanic productivity, eustatic sea level and tectonics that we have barely begun to document and understand. □

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## Astrochemistry

# Aluminium clues to the formation of the Solar System

from Donald D. Clayton

WHEN groups in Australia and at the California Institute of Technology first showed that aluminium-rich minerals from the Allende meteorite contained more  $^{26}\text{Mg}$  than expected, it was argued that a supernova must have exploded beside the forming Solar System. The argument went that the explosion peppered the solar cloud, from which Allende formed, with the short-lived and radio-active  $^{26}\text{Al}$ , and that the excess  $^{26}\text{Mg}$  in the meteorite is the result of decay of  $^{26}\text{Al}$  within the present meteoritic minerals. This conclusion has now been shaken by the detection of surprisingly

large amounts of radioactive  $^{26}\text{Al}$  in the interstellar medium. As a result, alternatives to both the theory of injection from an exploding supernova and the theory of decay within the actual meteorite are being seriously considered.

The original detection of  $^{26}\text{Al}$  — the first ever detection of interstellar radioactivity — was made by the HEAO 3 spacecraft (Mahoney, W.A. *et al. Astrophys. J.* **286**, 578; 1984) which recorded a measurable flux of 1,809 keV gamma rays striking the Solar System. These rays are a well known electromagnetic radiations product of the beta decay of the  $^{26}\text{Al}$

nucleus. HEAO 3 measured about 4.8 such gamma rays per square meter per second, coming from the general direction of the centre of our Galaxy, but at an unknown distance. Any lingering doubts about the reality of this astonishing discovery have since been removed by a gamma-ray spectrometer on board the Solar Maximum Mission satellite which, since February 1980, has taken unintentional periodic looks at our galactic centre. The spectrometer team (Share, G. H. *et al.*, submitted to *Astrophys. J.*) confirm that there are about 40 gamma rays per square metre per second coming from the general direction of the galactic centre, so that it can now be asserted without reasonable doubt that a source of radioactive  $^{26}\text{Al}$  lies in that general direction.

In an analysis of the magnitude of this gamma-ray flux and its implications for both the origin of the elements in explosions of stars and for the origin of the Allende minerals (Clayton, D. *Astrophys. J.* **280**, 144; 1984) I came to two unexpected conclusions. First, if the  $^{26}\text{Al}$  is spread uniformly throughout the interstellar gas, its concentration of about 10 parts per million of aluminium is rather close to that provided by Allende minerals, suggesting that it is unnecessary to invoke the injection of  $^{26}\text{Al}$  into the forming Solar System by a supernova trigger. Second, supernova explosions are not adequate to maintain the average level of observed interstellar radioactivity, so that nova explosions or gas streaming away from giant stars are the more likely origins of the radioactive aluminium now found in the interstellar medium. This also argues against the presence of a special supernova trigger to Solar System formation. A.G.W. Cameron, an early advocate of the supernova trigger theory, has given that idea up. He now argues (*Icarus* **60**, 416; 1984) that wind from an asymptotic giant branch star of about one solar mass carried ample  $^{26}\text{Al}$  along with it and participated hydrodynamically in the formation of a molecular cloud core wherein the Sun formed. A similar argument could be constructed with winds from Wolf-Rayet stars, which are also now expected to be enriched in fresh  $^{26}\text{Al}$  (Dearborn, D. & Blake, J. *Astrophys. J. Lett.* **288**, L21; 1985).

Interpretation of the excess  $^{26}\text{Mg}$  in Allende aluminium-rich minerals depends on the correct interpretation of the  $^{26}\text{Al}$  gamma-ray line, which requires better information on its angular distribution. Because of their wide viewing angles and serendipitous measurements, the HEAO 3 and Solar Maximum Mission teams have been reluctant to be very precise about the angular distribution. Although they describe it as being 'consistent with' radioactivity concentrated in the galactic plane and with peak intensity near the galactic centre, they stop short of claiming that the data requires that interpretation.

An alternative explanation invokes a



supernova explosion ten thousand years ago and about 20 parsecs from the Sun. This could happen to have been toward the general galactic centre, and would occupy a large circular area on the sky (perhaps a  $30^\circ$ – $90^\circ$  cone). But it would be most unlikely that the centre would lie in the plane of the Galaxy. Only if the observing teams can successfully show that the latitude distribution is narrow and centered on the plane, will we be forced to accept the observed concentration of  $^{26}\text{Al}$  as a general feature of the gas. In what follows, however, let us assume that the  $^{26}\text{Al}$  is a general galactic feature rather than a local one.

At  $10^{-5}$ , the interstellar ratio of  $^{26}\text{Al}/^{27}\text{Al}$  lies squarely between, but distinct from the ratio of  $5 \times 10^{-5}$  for some, but not all, aluminium-rich Allende minerals and that of less than  $10^{-6}$  could be maintained there by supernova explosions. The excess of  $^{26}\text{Al}$  in Allende minerals is therefore still too large to be interpreted as being the average interstellar concentration. Thus, if the present  $^{26}\text{Al}$  decayed in the present Allende minerals, there must have been some source of  $^{26}\text{Al}$  enhancement in the solar cloud as it collapsed to form the Solar System. This is the reinterpretation that Cameron now advances.

There are, however, new doubts that the  $^{26}\text{Al}$  was ever 'alive' in Allende, as I discussed at the 16th Lunar and Planetary Science Conference in Houston, during March 1985. Whatever objects serve as the source of the  $^{26}\text{Al}$  currently observed by gamma radiation, they are necessarily

ejecting  $4M_\odot$  of it per million years into the interstellar medium. By contrast, supernova eject  $24M_\odot$  of the new stable aluminium, and stars reinject  $60M_\odot$  of old stable aluminium over the same million years. If all of these ejecta condense into refractory aluminium-rich solids as they leave their respective sources, and if the  $^{26}\text{Al}$  decays to  $^{26}\text{Mg}$  within the resulting mixture of well mixed dust grains, the ratio of  $^{26}\text{Al}$  within the aluminium-rich interstellar dust will be 0.05, fully a thousand times greater than the correlation in the meteoric aluminium-rich minerals. This high interstellar correlation of excess  $^{26}\text{Mg}$  to Al could not have been totally removed by evaporation, because the Allende minerals carry other isotopic anomalies that demonstrate that they were not evaporated totally at any stage prior to their assembly. Thus these arguments indicate that the  $^{26}\text{Mg}$ –Al correlation observed in Allende minerals may be a manifestation of a cosmic chemical memory; the observed concentration of interstellar  $^{26}\text{Al}$  necessarily creates such a large interstellar  $^{26}\text{Al}$  anomaly that the special chemistry invoked for the  $^{26}\text{Mg}$  daughter is no longer required by a fossil theory. The challenge now to mineral chemists is to demonstrate how this memory survives the process of the formation of minerals and the substantial extraction of magnesium from them. □

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the spring and summer. Such results make it seem more likely that the smoke will last long enough and move far enough south to affect seriously the tropics and the Southern Hemisphere.

In mid-northern latitudes, full-blown nuclear winter effects will probably occur but, as this is the zone where most damage will be done by the direct effects of the nuclear explosions, this 'lofting' effect will be irrelevant. But as the smoke spreads southwards it will stop the solar heating of continental land surfaces that drives the south-west monsoon, halting the rainfall necessary for survival over most of southern and south-east Asia. This could be far more disastrous than temperature decreases in the tropics of  $10$ – $15^\circ\text{C}$ , even though rice is particularly sensitive to this degree of cooling.

Further south, rainfall over mainland Australia is expected to decrease, initially because of strengthening of the descending arm of the Hadley Circulation in the southern sub-tropics, and later from a general decrease in the hydrological cycle. A pre-'nuclear winter' study by B. Hunt (*Mon. Weather Rev.* **81**, 3677; 1976), where an 18-level primitive equation model was run with the solar insolation turned off, showed a rapid decline in the water vapour content of both the atmosphere and the eddy kinetic energy (strength of the synoptic weather disturbances). As Australian agriculture is mostly limited by precipitation, this effect will have a major effect on productivity, whereas the relatively modest temperature reductions may be less serious. New Zealand and Tasmania, on the other hand, are further south, so temperature is more limiting and even modest cooling may significantly reduce production.

Australia, New Zealand and Argentina currently have food surpluses, so that even large reductions in production may not lead to mass starvation in these countries, unlike much of southern Africa and the tropics. Thus, the greatest impact of the 'nuclear winter effect' may result from reduced rainfall in the highly populated countries of Asia and Africa rather than in countries in the mid-northern latitudes.

Several major uncertainties have been given more adequate treatment in recent, as yet unpublished, work discussed during the workshop. Areas still to be investigated are the rates of smoke washout in the initial plume-rise phase, the optical properties of aged smoke (especially at infrared wavelengths) and the possible smoke removal processes in the stratosphere. Better knowledge of effects on the Southern Hemisphere also requires computer simulations to be run for longer periods and to consider the effects of a war fought in the northern winter. □

## Nuclear war

# Poor outlook for tropical weather

from A. Barrie Pittock

THE most important environmental effect of a major nuclear war in the Northern Hemisphere may be caused by reduced rainfall in the tropics and subtropics. This conclusion was reached at a recent conference\* and will be incorporated in a report later this year (see *Nature* **315**, 534; 1985).

SCOPE (the Scientific Committee on Problems of the Environment) has run a series of scientific workshops, this being the first to be held in the Southern Hemisphere. New results from computer simulations were reviewed, incorporating explicit modelling of smoke dispersion from target areas in North America, Europe and the Soviet Union, and fully interactive smoke (that is, smoke that modifies the atmospheric temperature and wind structure and is then moved around by the modified circulation). This

overcomes two of the major weaknesses of the earlier simulations, which assumed an initial uniform smoke distribution over Northern Hemisphere latitude zones and did not allow for smoke movements.

A common feature of the results from simulations performed by the three different laboratories in the United States (Los Alamos, Livermore National Laboratories and the National Center for Atmospheric Research) and in the Soviet Union, was that smoke was 'lofted' from its initial height of injection to around  $10$  to  $20$  km in altitude and then moved south, because of heating by absorption of solar radiation. This effect occurs only in the northern spring and summer seasons when the solar radiation is sufficient to cause the lofting to take place. In winter the smoke tends to move towards the Arctic and is more quickly removed.

This lofting effect and the increased stability of the lower atmosphere (which reduces precipitation) quickly separates the smoke from the washout processes in

\* The workshop on the environmental effects of nuclear war was held in Melbourne, 28–29 March 1985, sponsored by the Australian National Committee for the Environment and the New Zealand Committee for the Scientific Committee on Problems in the Environment.

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## Physiological role of $\text{Ca}^{2+}$ transport by mitochondria

SIR—We cannot but whole heartedly agree with D.J. Miller<sup>1</sup> that the systems which transfer calcium across the inner membrane of mitochondria are of physiological importance. However, we are very surprised that he completely ignores the strong evidence<sup>2-8</sup> which indicates that the major role of these systems is to regulate the concentration of  $\text{Ca}^{2+}$  within the mitochondrial matrix rather than, as Miller suggests, the concentration of  $\text{Ca}^{2+}$  in the cytoplasm. When extramitochondrial  $\text{Ca}^{2+}$  is raised within the physiological range, a parallel increase in intramitochondrial  $\text{Ca}^{2+}$  will occur and this may activate three important dehydrogenases which occupy key regulatory sites in oxidative metabolism, namely pyruvate dehydrogenase, NAD-isocitrate dehydrogenase and 2-oxoglutarate dehydrogenase. In this way, the formation of reducing power for oxidative phosphorylation can be accelerated under the very conditions where there is likely to be an increased requirement for ATP. Increases in cytoplasmic  $\text{Ca}^{2+}$  generally stimulate energy-requiring processes such as muscle contraction, secretion or ion-pumping. It is worth emphasizing that these key dehydrogenases have so far only been found to be sensitive to activation by  $\text{Ca}^{2+}$  in vertebrate tissues<sup>9</sup> and only mitochondria from vertebrate tissues appear to take up  $\text{Ca}^{2+}$  at an appreciable rate at  $\mu\text{Molar}$  concentrations.

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## The electrostatic charge really is on the surface

SIR—A. A. Berezin recently discussed the question: "What will be a minimum energy configuration of a system of  $N$  equal point charges  $q$  placed inside a circle?" (see *Nature* **315**, 104; 1985). He considers two configurations, one (A) with all  $N$  charges equally spaced around the circumference and the other (B) with one

charge at the centre and the remaining  $N-1$  equally spaced around the circumference. Assuming a three-dimensional Coulomb potential  $q^2/r$  between pairs of charges, he finds numerically that  $W(A) < W(B)$  for  $N \leq 11$  but that for  $N \geq 12$  (up to at least  $N=400$ )  $W(A) > W(B)$ , where  $W$  represents the total electrostatic energy of the system. Whilst this answer to the question posed is doubtless true and has some curiosity value, the subsequent suggestion that these results provide an example where charges in a conductor at equilibrium will not reside on its surface is quite wrong.

The fallacy lies in the use of three-dimensional electrostatics for a two-dimensional system. Berezin's circle should properly be regarded as a thin disk and then the charge at the centre of the circle in B is actually at the centre of one of the flat surfaces of the disk. Thus, in the correct three-dimensional interpretation, all of the charges are on the surface of a thin conducting disk for both configurations A and B. (When  $N$  is very large, most of the charges will, at equilibrium, reside on the flat surfaces.)

It is of some interest to consider whether any anomalous behaviour arises in the solution of the above problem when it is regarded as strictly two-dimensional. The relevant Coulomb potential is now  $-q^2 \ln(r/L)$ , where  $L$  is an arbitrary length determining the zero of the potential. It is convenient to choose for  $L$  the radius of the circle. Then a straightforward analytic calculation shows that  $W(B) - W(A) = (q^2/2)[N \ln N - (N-1) \ln(N-1)] > 0$  for all  $N \geq 3$ . Thus, in the strictly two-dimensional problem the 'obvious' result is correct and our faith in classical electrostatics is restored.

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## The complexity and stability of ecosystems

SIR—I read Pimm's<sup>1</sup> article on stability and complexity within ecosystems with great interest. I would not, as an old-fashioned geologist-palaeontologist, pretend that I understood all of the logic involved, nor that I had been able to follow all of the mathematics employed in the May<sup>2</sup> and MacArthur papers to which Pimm refers. There is completely independent evidence from the fossil record supporting Pimm, as well as the earlier work by May<sup>2</sup> to which Pimm refers. Ecologically complex structures such as reef complexes appear historically to have been less stable than level bottom communities. If one concludes that the global marine ecosystem has been subjected to stress sufficient to generate large-scale extinction events, such as oc-

curred at the end of the Permian, then it follows that a comparison can be made between the resultant behaviour of the more simple level bottom communities and faunas as contrasted with the trophically far more complex reef community complexes. The nature of the stress(es) is (are) unknown, but it is reasonable to conclude that the trophically different community types reacted differently. Should one or the other, or at least elements of one or the other, survive such a major environmental perturbation it could be concluded that the surviving items were more stable.

There are five major intervals during the Phanerozoic when reef community complexes were in existence—including the present (Fig. 3 in ref. 3). In the Lower Cambrian some of the archaeocyathids occur in what many feel are reef community complexes. Such complexes disappear totally at the end of the Lower Cambrian concurrent with an important extinction event, whereas some level bottom taxa, particularly among the trilobites, survive to later radiate in the Middle Cambrian. The second interval of reef community complex is present in the Middle and Upper Ordovician. It disappears totally in the major, terminal Ordovician extinction event, together with much, but by no means all, of the level bottom fauna grouped into many communities. A third reef community complex, with antecedents differing from those of the Middle and Upper Ordovician, appears about one-third of the way through the Upper Silurian and persists until the middle of the Upper Devonian. The major mid-Upper Devonian extinction totally wipes out the reef community complex together with the majority of the level bottom taxa and many of the communities. A fourth reef community complex occurs in the Upper Carboniferous and Permian, derived from totally different antecedents (presumably, as always, level bottom taxa). The terminal Permian extinction event eliminates the Permo-Carboniferous reef community complex, and the bulk of the level bottom taxa and communities. The level bottom survivors ultimately provide the material for a re-radiation which is responsible for the different reef community taxa of the later Triassic.

There are significant taxonomic differences between the Triassic reef taxa and those of the Jurassic-Cretaceous, which suggests that the terminal Triassic extinction event was involved here. At the end of the Cretaceous the tropical-subtropical reef community complex was almost totally wiped out, and it does not reappear in any abundance until the Eocene. The reef community complex then persists until the present, although there are some major changes, such as the addition of the algal ridge complex in the Miocene. Large numbers of coeval



Jurassic–Recent level bottom taxa persist to the present.

In view of the above it is likely that following severe environmental stress capable of generating major extinction events preserved in the fossil record the reef community complex normally is eliminated *in toto*, but that elements, sometimes a large percentage, of the level bottom fauna and its communities persist. Supporting this conclusion is the fact that during the highly provincial Ordovician, Permian, and later Cretaceous, the biogeographically distinctive reef community complexes in different parts of the world terminate at the same time.

I suspect that other trophically complex community types such as pelmatozoan thickets, bryozoan thickets and sponge forests, behave in a fashion similar to the reef community complex rather than to the trophically simpler level bottom communities.

It seems clear that the geological-palaeontological evidence strongly supports Pimm<sup>1</sup>, May<sup>2</sup>, and the others in their conclusions based on a variety of non-geological evidence.

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## Icosahedra in aluminium / manganese alloy

SIR — The experimental observations of regions of icosahedral order in a splat-cooled alloy of Al/Mn<sup>1,2</sup> have excited considerable comment on a novel type of structure which hitherto had been purely conjectural<sup>3</sup>. One way of considering the proposed structure is as the packing of two kinds of rhombohedral units cells, both having the same cell side but one being acute with  $\alpha=63.43^\circ$  and the other obtuse with  $\alpha=116.57^\circ$ . These are repeated according to what has become known as the three-dimensional Penrose pattern, and in a ratio of  $\tau$  acute units to each obtuse unit, where  $\tau$  is the golden number 1.618... The pattern is intrinsically recursive or hierarchic. I wish to point out further similarities with structures already known. There are at least three types of structures entailing local icosahedral symmetry which may be extended hierarchically for various ranges through the material, according to the balance of the demands of local and of long-range ordering. These include:

(1) Close-packed metal structures where, as pointed out by Frank in 1952<sup>4</sup>, 12 spheres will pack icosahedrally around a thirteenth which has 95% of their diameter. This is well known in alloys, most clearly in the structure<sup>5</sup> of Mg<sub>32</sub>(Zn,Al)<sub>49</sub>

which builds four icosahedral shells before the structure goes periodic. Most atoms in it have local icosahedral coordination. Probably the driving force behind the structure found by Shechtman *et al.* is of this type. We might note that “decagonal snowflakes” seem first to have been reported by Adam and Hogan and co-workers<sup>6,7</sup> for the corresponding Al/Fe alloy, but were then not so fully characterized.

(2) The boron compounds, summarized graphically by Sullenger and Kennard<sup>8</sup>, where many boron compounds, notably  $\alpha$ - and  $\beta$ -boron and B<sub>12</sub>C<sub>3</sub>, have rhombohedral unit cells with  $\alpha$  about  $65.5^\circ$ , closely corresponding to the acute quasi-unit-cell of the Penrose pattern. Here the symmetry results from the icosahedral clusters of boron atoms produced by the directed bonding requirements of boron to which Berezin<sup>9</sup> has drawn attention. It might be noted that in  $\beta$ -boron the packing is hierarchic, 12 icosahedra being themselves packed icosahedrally around a thirteenth.

(3) Tetrahedrally connected networks, where dodecahedral cages can also appear because of the closeness of the tetrahedral angle of  $109.47^\circ$  to the vertex angles of a pentagon ( $108^\circ$ ). These occur in clathrasils (cage silicates) and in clathrate hydrates.

Using the structural elements which occur in the zeolite ZSM-39<sup>10</sup> and in the type I gas hydrate<sup>11</sup> we have constructed hypothetical unit cells which would correspond to those required for the Penrose pattern. The acute unit cell is composed of Si–O polyhedra as in the ZSM-39 sheets and these polyhedra are the duals of those in the  $\beta$ -boron structure. The unit cell edges consist of alternating dodecahedra sharing faces (two per edge, so that the cell edge length is 4.45408 times the edge length of the dodecahedron). The corresponding obtuse cell consists of the same faces (arranged with  $\alpha=116.57^\circ$  instead of its supplement) but has, at its centre, a polyhedral cage of 18 faces with two dodecahedral caps (of six pentagons each) and a belt of six hexagons equatorially.

Next to this cage the dodecahedra are dislocated and have each two hexagonal and two square faces as well as eight pentagonal faces. Thus, the Penrose pattern reported for alloys might also occur in silicates or hydrates, the dodecahedra of the open tetrahedral structure being dual to the icosahedra of the close-packed metallic structures.

The same two acute and obtuse units may also be assembled in various crystalline ways with, for example, the ratio of 5 acute to 3 obtuse. In view of the occurrence of the two kinds of unit cells in the numerical ratio  $\tau$  in the Penrose pattern, it might be timely to look back at the electron compounds of Hume-Rothery<sup>12,13</sup>, some of which are locally icosahedral, whose electron/atom ratios are 21/13 (these often with an atomic ratio of 8/5 as for Cu<sub>5</sub>Zn<sub>8</sub>) and 3/2 for CuZn, which

might seem to be convergents to the golden number (and also 7/4 which is not), to see where else the Fibonacci series may be detected.

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## Loess in the Bering Land Bridge

SIR — In his *News and Views* article on the Bering Land Bridge, Colinvaux<sup>1</sup> reported that the *yedoma* deposits of Siberia — long described as alluvium or lacustrine silt — are actually loess, emplaced by wind action when “dry, cold and windswept” conditions prevailed in that part of Siberia. The climatic consequences of this conceptual change are profound and Academician Shilo and co-workers are to be congratulated in bringing about this revolution. Once an idea is in place (for example that a particular deposit is lacustrine silt), it can be very difficult to change; witness the remarkably long time it took to recognize the widespread loess in southern England. But, if the Siberian story is to be fully told there should be a nod of acknowledgement in the direction of Troy Péwé and his proposals that Siberian “silt” were loess<sup>2,3</sup>. Péwé and Journaux, admittedly working west of the immediate Bering Strait area, showed that loess was widespread in Yakutia and made a good case for the existence of significant Siberian loess deposits<sup>4</sup>.

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## Scientific Correspondence

Scientific Correspondence is intended to provide a forum in which readers may raise points of a rather technical character which are not provoked by articles or letters previously published (where Matters Arising remains appropriate).

# Refraction scattering as origin of the anomalous radar returns of Jupiter's satellites

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*Three satellites of Jupiter, Callisto, Ganymede and Europa, give anomalous radar returns. We show here that this behaviour would arise as a consequence of 'refraction scattering' rather than the familiar 'reflection scattering' and discuss mechanisms that lead to the predominance of such scattering on these satellites.*

THREE of the Galilean satellites of Jupiter, Callisto, Ganymede and Europa, have been shown to have anomalous radar reflection properties in several respects (refs 1-3; D. B. Campbell and S. J. Ostro, in preparation). Although all other planetary bodies that have been investigated by radar, including many different areas on the Earth, give a reflection of a circularly polarized radio wave in which the sense of polarization is reversed, these jovian satellites return a wave that shows predominantly the same sense as the incident one: the shorter the wavelength, the more similar the sense of the waves. If a linearly polarized illumination is used, fully 50% of the energy is returned in the orthogonal polarization, whereas the normal for other bodies is <10%.

Although all other planetary bodies seem to have a total radar cross-section (measuring the sum of returned power in two orthogonal polarizations) of the order of  $0.1 \times$  the geometrical cross-section, the total cross-section at 12.6-cm wavelength for the galilean satellites are 2.6 for Europa, 1.5 for Ganymede and 0.6 for Callisto<sup>4</sup>.

All other planetary bodies give a quasispecular return (glare) from the subearth point, but no such effect occurs for the three galilean satellites in the wavelength region in which they have been studied (3.5-70 cm). In fact, the scattering from these satellites is intermediate between that giving a uniformly bright disk and the slight limb-darkening associated with Lambert scattering<sup>4</sup>.

In addition to their remarkable visual appearance<sup>5,6</sup>, Callisto, Ganymede and Europa are best characterized as bizarre radar objects. Ostro<sup>4</sup> discusses two possible mechanisms which might explain the peculiar polarization properties. One, which is discarded as unlikely, involves double reflection within hemispherical craters, whereas the other involves multiple internal total reflection on flat boundaries between ice and less dense ice (crushed ice, possibly snow). The polarization results, studied by Monte Carlo calculations, involve boundaries of random and uncorrelated orientations. There is no good explanation of the enormous cross-section in this model.

We explore here the alternative possibility that the effect results from what we term 'refraction scattering', a type of scattering which has received little attention and which, has not been encountered previously in radar work with solid materials.

In refraction scattering, one measures a wave that penetrates into the body and whose propagation vector is bent as a result of gentle variations of the refractive index in the medium. In the case of sufficiently large variations of the refractive index, the penetrating wave may be deflected so as to emerge again from the surface. In the geometrical optics limit, the polarization of the wave is retained and we shall show that the unusual

polarization properties of the three satellites are then reproduced. For refraction scattering to be dominant one requires: (1) The surface reflection must be small. This requires that the surface material has a low refractive index,  $n$ , or that there is a 'matching' transition layer on the surface; (2) the bulk medium has sufficiently low absorption of radio waves to allow long pathways within the medium; (3) the patterns of the variations in  $n$  are such as to redirect the propagation vectors by large angles, including  $180^\circ$ ; (4) the gradients of  $n$  are sufficiently small so that internal reflections will not have a dominant influence.

We describe below internal refractive index structures that satisfy the above four requirements and can reproduce naturally all the bizarre radar properties of the three outer galilean satellites.

## Internal refraction

The study of the propagation is based on geometrical optics. A set of equations which describes the rays is:

$$\frac{d\vec{r}}{ds} = \vec{s} \quad (1)$$

$$\frac{d\vec{s}}{ds} = \vec{s} \times \left( \frac{\nabla n}{n} \times \vec{s} \right) \quad (2)$$

where  $s$  = arc length along ray and  $\vec{s}$  = unit vector along ray. If the medium is horizontally stratified and  $n$  is higher in the medium than outside, the angle between the ray direction  $\vec{s}$  and the vertical must always be less there than at incidence. Therefore, the ray cannot emerge in the return direction and it is necessary that the surface material is laterally inhomogeneous. Surfaces of constant  $n$  might have an appearance as shown in Fig. 1.

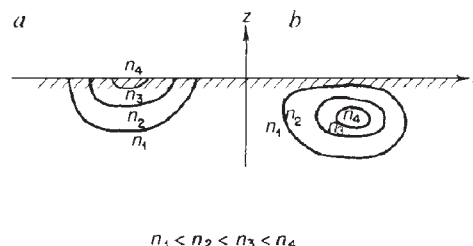


Fig. 1 Contours of constant refractive index: a, partly buried; and b, fully buried scatter.



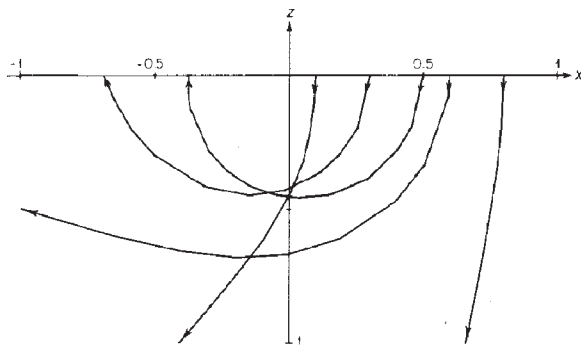


Fig. 2 Rays in gaussian refractivity profiles. Normal incidence.

What seems to be required are convex surfaces of constant  $n$  surrounding a region of higher  $n$ . Further, the curvature of the rays  $|\nabla n/n \times \vec{s}|$  must be comparable with the curvature of the equi-refractive index surfaces. These regions of excess refractive index may be quasi-spherical or ellipsoidal, or they may be torus or cylinder shaped with quasi-circular or ellipsoidal cross-sections. Whatever the case may be, such regions, possessing a sufficiently large total range of  $n$ , are capable of bending the incoming rays so that they emerge from the surface in all different directions, including the backscatter direction.

We have numerically constructed rays on the basis of equations (1) and (2) for surfaces of several types of  $n$ , spherical, ellipsoidal and cylindrical, where  $n$  varies either as a cosine, a gaussian or according to the function given below. The refractive index was taken to vary from  $n_0$  at the centre to the value  $n_1$  of the background medium at a distance from the centre,  $r$ . Various degrees of submersion of the blobs were considered. The special function mentioned is of the form:

$$n(x, z) = \frac{n_0}{1 + (x^2 + (z - z_c)^2)/a^2}$$

$$\text{when } \begin{cases} z < 0 \\ \sqrt{x^2 + (z - z_c)^2} < a\sqrt{n_0/n_1 - 1} \end{cases} \quad (3)$$

$$= n_1 \quad \text{when } \begin{cases} z < 0 \\ \sqrt{x^2 + (z - z_c)^2} > a\sqrt{n_0/n_1 - 1} \end{cases}$$

$$= 1 \quad \text{when } z > 0$$

An example of rays traced in a gaussian profile with  $z_c = 0$  (Fig. 1a),  $n_0 = 3.5$ ,  $n_1 = 1$  and  $L_x = L_z = 0.4$  (the  $e$ -folding distances in the  $x$  and the  $z$  directions, respectively) is shown in Fig. 2, for normal incidence.

We find no evidence of any qualitative difference between the different models and, therefore, discuss the special case as defined in equation (3) in greater detail, as this is amenable to straightforward analytical treatment. This particular profile, referred to as Maxwell's 'fish eye', has been discussed elsewhere<sup>7</sup>.

Consider a spherical blob of excess  $n$  of this type fully embedded in a medium of refractive index  $n_1$ . We wish to determine the deviation angle between the incoming and existing rays as a function of  $x$  (analogous to the impact parameter in collision theory). This relationship can best be derived by reference to Fig. 3. Suppose the radius where the excessive refractive index equals that of the surrounding medium (that is,  $n_1$ ) is  $r_1$ , and suppose the  $z$ -axis is aligned with the incoming ray. The ray enters at A. In such refractivity distributions the curvature of the ray is constant and the ray must describe a circle, the centre of which must lie on a line AE normal to the  $z$ -axis. A ray going through point A which is a distance  $r_1$  from the centre will cross the diameter through A at a point B on the opposite side of C at a distance  $BC = r_2$ . In this particular distribution

one has:

$$r_1 r_2 = a^2$$

$$a = r_1 / \sqrt{n - 1} \quad (4)$$

$$n = n_0 / n_1$$

The centre of the circular ray is found at the intersection of the bisector of AB with AE, and is thus D. From the construction of Fig. 3 we find that the total deflection angle  $\theta$  is given by:

$$\theta = 2 \arctan \frac{2(n-1) \cdot x \cdot \sqrt{1-x^2}}{n-2x^2(n-1)} \quad (5)$$

where  $x \Rightarrow x/r_1$  is the normalized 'impact parameter'.

Figure 4 shows the deflection angle for this particular model plotted against normalized  $x$  for various values of  $n$ . The actual values of  $x$  required to give backscatter (that is,  $\theta = 180^\circ$ ) are given by:

$$x_{180} = \sqrt{\frac{n}{2(n-1)}} \quad (6)$$

and are plotted in Fig. 5a. It is clear from this diagram that the rays that are turned back by  $180^\circ$  enter and leave the scatterer near the circumference where  $n$  is small. As they will never go through the centre of the scatterer it is not necessary that  $n$  actually assumes the value  $n_0$  for backscattering to occur, but it is sufficient that  $n$  increases to  $n_{\max}$ , which it assumes at that point of the ray where it is the closest to the centre of the scatterer. The actual value of this  $n_{\max}$  can be shown to be:

$$n_{\max} = \frac{n}{n - \sqrt{n(n-2)}} \quad (7)$$

Figure 5b shows  $n_{\max}$  as a function of  $n$ . It is clear from this diagram that the actual values of  $n(r)$  need not in practice reach the peak value at the centre of the scatterer to be able to turn the rays round by  $180^\circ$ .

It can be seen from Fig. 4 that there is a substantial range of  $x$  which, for normal incidence, turns the rays back into the hemisphere they came from. If the inner and the outer limits for  $x$  are denoted by  $x_{90}$  and  $x_{270}$ , the fractional energy returned into the hemisphere centred on the incoming wave is  $x_{270}^2 - x_{90}^2$ . This fraction is plotted as a function of  $n$  in Fig. 5c.

Finally, we consider the radar backscattering cross-section of one of these spherical enhancements of refractivity. This cross-section is defined as:

$$\frac{\text{Power scattered within } \Delta\Omega}{\text{Incident power density}/4\pi} = \sigma \quad (8)$$

In the geometrical optics approximation discussed above we have found a unique mapping between the area  $\Delta A$  of the wave front and the solid angle  $\Delta\Omega$  onto which this power is scattered. Therefore, the cross-section  $\sigma$  can be expressed as:

$$\frac{S \cdot \Delta A(\Delta\Omega)/\Delta\Omega}{S/4\pi} = 4\pi \cdot \frac{\Delta A}{\Delta\Omega} \quad (9)$$

For an arbitrary direction we have:  $\Delta A = x \cdot \Delta x \cdot \Delta\psi$  and  $\Delta\Omega = \Delta\theta \cdot \sin\theta \cdot \Delta\psi$ . Hence we obtain:

$$\sigma = 4\pi \cdot \frac{x}{\sin\theta} \cdot \frac{\Delta x}{\Delta\theta} \quad (10)$$

From equation (5) it is easy to show that  $\Delta x/\Delta\theta$  remains finite for backscatter, and because  $x$  remains near unity  $\sigma$  becomes infinite as  $\sin\theta \rightarrow 0$ . This apparent paradox is resolved if we remember that  $\Delta\Omega$  cannot approach zero with  $\Delta A$  because diffraction eventually starts  $\Delta\Omega$  increasing again for small  $\Delta A$ . We should also note that rays entering and leaving along a circle of radius  $x$  will only occur when there is perfect spherical

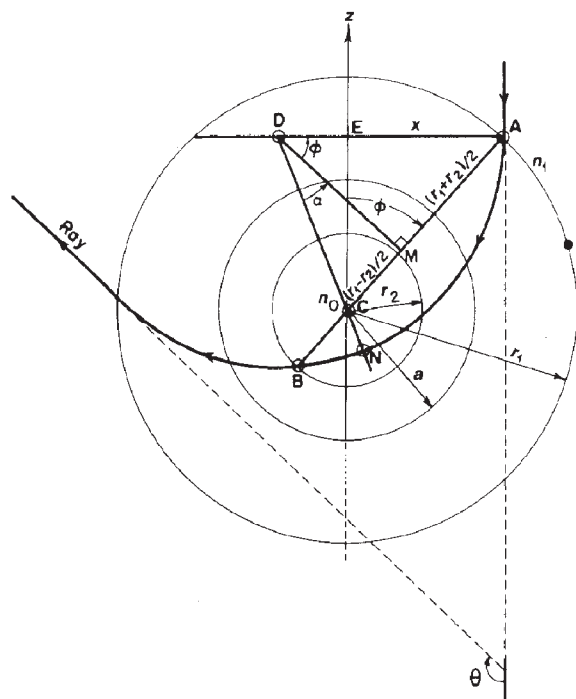


Fig. 3 Derivation of the deflection angle for 'fish eye' distribution of  $n$ .

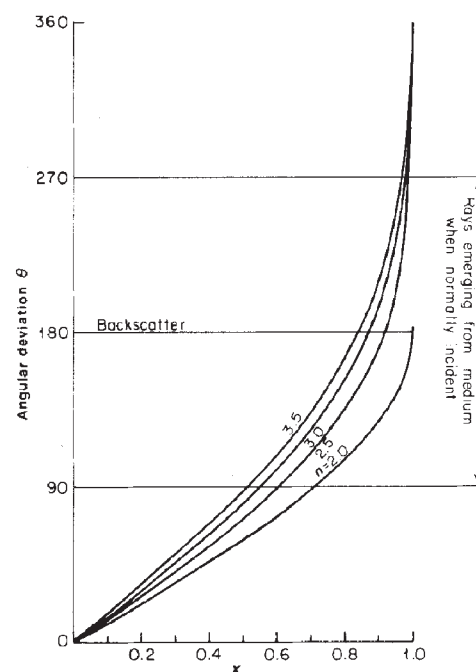


Fig. 4 Angular deviation of rays plotted against 'impact parameter'  $x$  with  $n = n_0/n_1$  as parameter.

geometry. As soon as we allow for deviations from the perfect spherical symmetry the circular ring will be broken up into disjointed areas and the tendency for backscatter probably decreased. Nevertheless, we suspect that there will also be a distinct preference for backscatter in the real surface.

## Application to observations

Let us now see how the concept of refraction scattering can be applied to explain the observed radar reflection properties of Callisto, Ganymede and Europa. We must assume that the presence of refractive scatterers is universal on these satellites. The description of the return as a refraction phenomenon, with little reflections from abrupt refractivity changes, implies that a circularly polarized wave will retain its polarization and emerge with the same sense of circular polarization, as required. Our model predicts circular polarization to be maintained fully, in the original sense, whereas the data show this to be only partly so. This may be due to residual surface backscattering, or some internal reflections and/or failure of the geometrical optics approximation to be perfect. Suppose we assume that the backscattering arises as a superposition of two distinct mechanisms, one being the refraction scattering and the other a more usual mechanism, which turns the incoming circular polarization completely into the opposite one in the same manner as an ideal reflecting surface. For a wavelength of 12.6 cm, Ostro<sup>4</sup> quotes for the ratio of 'same circular' (SC) to 'orthogonal circular' (OC) values of 1.5 for Europa and Ganymede and 1.2 for Callisto. It follows that 60% of the power returned from Europa and Ganymede and 55% from Callisto must result from refraction scattering.

The linear polarization results follow from the refraction model as follows: suppose we consider a ray in the  $xz$  plane as in Fig. 3. If the linearly polarized E-field is normal to the  $xz$  plane, the emerging E-field will be in the same direction as the incoming field. If, however, the E-field is in the incidence plane  $xz$ , it will emerge in that plane, but  $180^\circ$  out of phase with a corresponding field normal to  $xz$ . This means that a field making

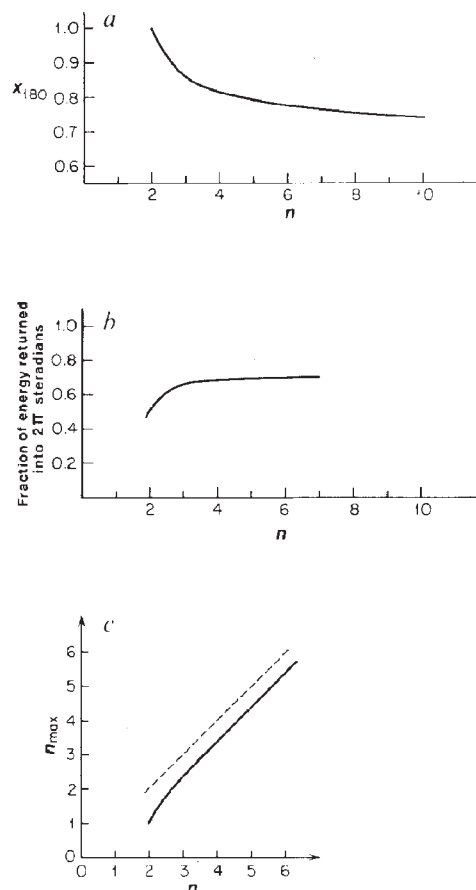


Fig. 5 a, Plot of  $x_{180}$  against  $n = n_0/n_1$ . b, Plot of maximum refractive index along a backscatter ray against  $n$  at the centre. c, Plot of fraction of normally incident energy emerging from the surface against  $n$ .



45° with the  $xz$  plane will emerge with its E-field normal to the incoming field. In general, for a field making an angle  $\gamma$  with the plane of incidence, one must have:  $E_{SL} = E_0 \cos 2\gamma$  and  $E_{OL} = E_0 \sin 2\gamma$  where the indices signify 'same linear' and 'orthogonal linear' as in ref. 4. If powers rather than fields add, and because  $\gamma$  must occur with equal probability between 0 and 360°, one obtains  $\sigma_{SL} = \sigma_{OL}$  or  $\sigma_{OL}/\sigma_{SL} = 1$ . Hence, our ideal model predicts complete depolarization, whereas the data show this ratio to be 0.5. In addition, the assumption of power addition may break down if there is coherency between rays entering and leaving individual blobs, as indeed there is if they are perfectly spherical. Again it seems reasonable to suppose that surface reflections, internal reflections and/or failure of the geometrical optics approximation to decrease the predicted polarization ratio from that of the model. If we imagine that the returned powers be split between refraction scattering and a reflection scattering mechanism as above, and try to reproduce the ratio of orthogonal linear to same linear of 0.47 for Europa and Ganymede and 0.55 for Callisto as observed at 12.6-cm wavelength by Ostro<sup>4</sup>, we conclude that 64% of the power returned from the former two can be ascribed to refraction scattering, whereas 70% must be assigned to that mechanism in the case of Callisto. For Europa and Ganymede the consistency is excellent between the deductions made from the linearly and circularly polarized data in view of the limited applicability of our arguments. For Callisto, however, there is an inconsistency between the deductions made, which we suspect results from the failure of the geometrical optics approximation to apply as well as it apparently does on Europa and Ganymede.

We have shown that refractivity inhomogeneities are capable of scattering a substantial fraction of incoming radiation back into space. Such behaviour is consistent with the low emissivity observed by de Pater<sup>8</sup>. The extremely high radar reflectivity is also compatible with the low emissivity and is probably augmented by a preferential backscattering of the individual scatterers, discussed above.

For refraction scattering, or for scattering by internal reflections, the case of normal incidence at the surface has no particular significance; this will account for the lack of a specular reflection highlight near the sub-earth point on these satellites.

Finally, the variation with wavelength referred to earlier receives a natural explanation, in that the geometrical optics approximation will become increasingly poor the larger the wavelength. If this is the explanation of the wavelength dependence, then the size of typical scatterers must be of the order of 1 m.

## Discussion

It is noteworthy that the anomalous properties occur just on the only three bodies that have been investigated, which are thought to be composed mainly of ices. Water ice, thought to be the dominant component, is a dielectric of very low dielectric loss, with  $n = 1.78$ . The other component of the surface material of these bodies is believed to be rock powder whose detailed dielectric properties are not yet known. Lunar rocks and rock powders have been extensively investigated<sup>9</sup> and have values of  $n$  ranging from 1.3 for loosely packed material to  $>3.2$  for the dense rock. If there is a tendency for rocks in the domain of the outer planets to be less oxidized, then substantially higher values for the refractivity are to be expected.

Therefore, the physical model we propose is one of a patch-

work of rock powders and ices in which  $n$  may vary from just over 1 to  $>3$ . The uneven distribution of these two components may have resulted from an initial accretion of layers of rock and ice particles, which, together with impacts of larger objects, would have turned a neatly layered system into one that is both laterally and vertically erratic. In particular, it may be that the overturning of material in an impact could create the torus-shaped structures referred to above. Also, impact on a loosely packed but cohesive matrix of rock powder left behind by evaporation of volatiles could be compacted and form the semi-spherical volumes of excess  $n$  discussed above. Evaporation of volatiles from the surface may have helped to concentrate rock powders. Outpouring of liquid water from deeper and warmer regions may have laid down 'clean' ice. Both these processes would contribute to the creation of a heterogeneous medium.

The requirement of low dielectric loss may well be satisfied because of the very low temperatures. Ice well below its melting point leads to absorption lengths of thousands of wavelengths. The lunar rocks gave results for the absorption length between 100 and 300 wavelengths. At the low temperatures of these icy satellites it is likely that the low dielectric loss of these rock powders would be maintained despite the presence of water in solid form. (At the higher temperatures prevailing on earth, hydrated rocks dominate and exhibit a high dielectric loss.)

The condition of low surface reflectivity may be met by having a very tenuous medium (therefore of low  $n$ ) in which the regions of excessive refractivity are embedded. If the medium is not so tenuous, it may be that a low density transition layer, as found on the Moon, can provide the proper match and serve to suppress the reflection at the surface. An extremely fluffy outer surface of rock powder or snow may have resulted from the partial evaporation of ices from the pore spaces in the material. In addition it may be that the interesting mechanism suggested by Spencer and Maloney<sup>10</sup>, which explains the migration of water on Callisto as sublimation and capture in cold traps, may have played a role in creating the heterogeneous conditions required. This mechanism alone would apparently create depletions in refractivity rather than enhancements, and, therefore, must have been modified by some other mechanism, such as impact cratering, to create the favourable conditions for refraction scatter to occur.

Also within the material, sharp discontinuities of  $n$  must be sufficiently rare for refraction to remain the dominant cause of backscatter. If rock powder and ices make up the bulk of the material, this implies that although variations of the mixing ratio of these two substances are responsible for the refraction, they are generally not too abrupt on the scale of the radio wavelengths used.

## Conclusions

We have shown that refraction scattering from convex regions of excess  $n$  has all the properties required to explain the observed radar returns from Callisto, Ganymede and Europa. We conjecture that the conditions which prevail on these satellites are probably ideal for the creation of the refractivity structure required to make refraction scattering important. Therefore, we conclude that refraction scattering is the most likely explanation for the bizarre scattering properties of these satellites.

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# Cloning, sequence and expression of two distinct human interleukin-1 complementary DNAs

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*Two distinct but distantly related complementary DNAs encoding proteins sharing human interleukin-1 (IL-1) activity (termed IL-1 $\alpha$  and IL-1 $\beta$ ), were isolated from a macrophage cDNA library. The primary translation products of the genes are 271 and 269 amino acids long, although expression in Escherichia coli of the carboxy-terminal 159 and 153 amino acids produces IL-1 biological activity.*

INTERLEUKIN-1, a protein produced by activated macrophages, mediates a wide range of biological activities, including stimulation of thymocyte proliferation via induction of interleukin-2 (IL-2) release, stimulation of B-lymphocyte maturation and proliferation, fibroblast growth factor activity and induction of acute-phase protein synthesis by hepatocytes<sup>1</sup>. IL-1 has also been reported to stimulate prostaglandin and collagenase release from synovial cells, and to be identical to endogenous pyrogen<sup>1</sup>.

The question of whether this array of activities resides in a single protein has excited much discussion. The conventional approach to its resolution has been to purify IL-1 activities from the culture supernatants of macrophages activated with lipopolysaccharide (LPS), but the results have been ambiguous. Apparently homogenous protein preparations display characteristic IL-1 activities, including IL-2 induction and pyrogenicity. However, many reports associate IL-1 with proteins of relative molecular mass ( $M_r$ ) ~15,000 and 30,000 and isoelectric points ~pH 5 and 7 (refs 1-8). These studies suggest that either a considerable amount of protein processing leads to the appearance of active polypeptides of differing sizes from a single protein, or that activities characteristic of IL-1 are shared by several different proteins.

To clarify these issues, we attempted to clone the gene(s) responsible for IL-1 activity using two different strategies. First, we screened macrophage complementary DNA clones by hybridization to macrophage RNA, followed by *in vitro* translation of the selected RNA and detection of IL-1 biological activity using a sensitive assay that measures its capacity to induce IL-2 synthesis by a cloned T-cell line<sup>9</sup>. Second, we purified to homogeneity a protein having IL-1 activity<sup>2</sup>, performed partial amino-acid sequence analysis, constructed an oligonucleotide probe based on this sequence and used the probe to screen a macrophage cDNA library.

These experiments led to the isolation of two distinct cDNAs encoding proteins with a characteristic IL-1 activity, defined by the induction of IL-2 synthesis by a T-cell line or by thymocytes. These proteins, which we term IL-1 $\alpha$  and IL-1 $\beta$ , showed only distant homology to each other. Both molecules seem to be synthesized as larger precursors that are processed to smaller forms. Here we discuss the relationship of these findings to recent reports of other IL-1 cDNAs<sup>10,11</sup>.

## Isolation of IL-1 messenger RNA

Optimal conditions for the production of IL-1 messenger RNA from peripheral blood monocytes were established. Initially, mRNA was injected into *Xenopus* oocytes and the supernatants assayed for biological activity. Low levels of IL-1 could be detected using an assay that measures the capacity of IL-1 to

stimulate IL-2 release from a phytohaemagglutinin-stimulated cloned murine lymphoma cell line (LBRM-33-1A5) (termed the 1A5 conversion assay)<sup>9</sup>. This assay is ~1,000-fold more sensitive than the standard IL-1 assays of direct thymocyte mitogenesis or co-mitogenesis<sup>2</sup>, and was used as the basis for the molecular cloning of IL-1. Stimulation of the monocytes with 10  $\mu\text{g ml}^{-1}$  *Escherichia coli* lipopolysaccharide (LPS) for 12 h gave the highest levels of IL-1 mRNA. Unstimulated monocytes contained little IL-1 mRNA.

Table 1 Identification of IL-1 $\alpha$  cDNA clones by hybrid selection of biologically active mRNA

| First screen |                       | Second screen |                       | Third screen |                       |
|--------------|-----------------------|---------------|-----------------------|--------------|-----------------------|
| Filter       | IL-1                  | Filter        | IL-1                  | Filter       | IL-1                  |
| Starting RNA | (U ml <sup>-1</sup> ) | Starting RNA  | (U ml <sup>-1</sup> ) | Starting RNA | (U ml <sup>-1</sup> ) |
|              | 1,195                 |               | 1.5 $\times 10^4$     |              | 1.8 $\times 10^4$     |
| 1            | ND                    | 2A            | ND                    | 2C1          | 257                   |
| 2            | 59                    | 2B            | ND                    | 2C2          | ND                    |
| 3            | ND                    | 2C            | 1,006                 | 2C3          | ND                    |
| 4            | 10                    | 2D            | ND                    | 2C4          | ND                    |
| 5            | ND                    | 2E            | ND                    | 2C5          | ND                    |
| 6            | ND                    | 2F            | ND                    | 2C6          | ND                    |
| 7            | ND                    | 2G            | ND                    |              |                       |
| 8            | ND                    | 2H            | ND                    |              |                       |
| 9            | ND                    | 11A           | ND                    |              |                       |
| 10           | ND                    | 11B           | ND                    |              |                       |
| 11           | 72                    | 11C           | ND                    | 11F1         | ND                    |
| 12           | ND                    | 11D           | ND                    | 11F2         | ND                    |
| 13           | ND                    | 11E           | ND                    | 11F3         | ND                    |
| 14           | ND                    | 11F           | 440                   | 11F4         | ND                    |
| 15           | ND                    | 11G           | ND                    | 11F5         | ND                    |
| 16           | 39                    | 11H           | ND                    | 11F6         | 1,072                 |
| Control      | ND                    | Control       | ND                    | Control      | ND                    |

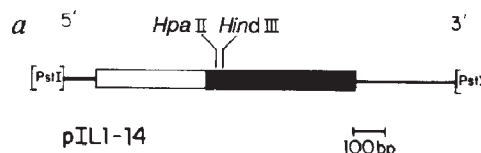
Single bacterial colonies were picked into LB-broth plus 15  $\mu\text{g ml}^{-1}$  tetracycline and grown overnight, then 48 1-ml cultures were combined and plasmid DNA isolated according to standard procedures<sup>27</sup> with the modification that the plasmid DNA was ribonuclease treated and separated from small- $M_r$  nucleic acids by chromatography on a Sephacryl S-400 column. 10  $\mu\text{g}$  plasmid DNA was digested with *Bam*HI, denatured and bound to nitrocellulose filters (Schleicher and Schuell)<sup>28</sup>. Each filter was hybridized to 20  $\mu\text{g}$  of LPS-stimulated macrophage RNA overnight at 50 °C in 50% formamide, 100 mM PIPES pH 6.8, 0.4 M NaCl, 10 mM EDTA, then washed 10 times at 65 °C in 1  $\times$  SSC, 0.2% SDS and five times in 0.2  $\times$  SSC at 65 °C. The bound RNA was eluted in boiling water containing 6.6  $\mu\text{g ml}^{-1}$  yeast transfer RNA and recovered by ethanol precipitation, then translated in rabbit reticulocyte lysates<sup>29,30</sup> and an aliquot of the lysate was assayed for IL-1 activity in the 1A5 conversion assay<sup>9</sup>. ND, not detected. No biological activity over the background control was observed at a sample dilution of 1:50. Control, 10  $\mu\text{g}$  pBR322 DNA was bound to the filter as a negative control.





**Fig. 2 a.** Restriction map of pIL-14. *Pst*I sites in brackets are those generated by the cloning procedure. Box, protein coding sequence; open box, N-terminal portion of the primary translation product removed during proteolytic processing; closed box, *M*<sub>r</sub> 17,500 biologically active portion of IL-1 $\beta$  isolated from activated macrophage supernatants. **b.** Nucleotide and predicted amino-acid sequence of the IL-1 $\beta$  primary translation product. Amino acids in capitals indicate those residues confirmed by protein sequencing analysis. Numbering of nucleotides is negative for the 5' non-coding region and positive from the presumed initiator methionine codon. Protein numbering begins at the initiator methionine. Underlined amino acids refer to the first cycle of different protein sequence analyses. Star, NH<sub>2</sub>-terminal residue of the *M*<sub>r</sub> 17,500 form of IL-1 $\beta$  (Ala 117). The *Hpa*II site was used in construction of plasmids used for expression of IL-1 $\beta$  (Fig. 4, Table 2).

**Methods.** **a.** A cDNA library was constructed from size fractionated, polyadenylated human macrophage RNA as described in Fig. 1a. A 62-nucleotide probe was constructed using the amino-acid sequence data corresponding to amino acids 133–158 (see below). The probe (5' ACTTGTTGTTCCATGCTTGGCCTTGCAGG TGCAGGGCTTTCAGTTCTGAGGGGCCGACAT 3') was labelled to high specific activity with T<sub>4</sub> polynucleotide kinase and [ $\gamma$ -<sup>32</sup>P]ATP and used to screen the library<sup>35</sup>. Positive colonies were analysed by restriction mapping using standard techniques<sup>12</sup>. **b.** Nucleic acid sequencing: Various restriction fragments of pIL-1-14 were cloned into M13mp18 or M13mp19 and sequenced as described in Fig. 1. Protein sequencing: amino-terminal sequencing: ~12  $\mu$ g (680 pmol) unmodified IL-1 $\beta$  was placed in a protein sequencer (Applied Biosystems Model 470A) and analysed according to manufacturer's specifications. Phenylthiohydantoin amino acids were identified by HPLC as described previously<sup>11</sup>. Residues 117–123 and 125–136 were identified and tryptic peptides prepared as follows: 10.5  $\mu$ g (600 pmol) IL-1 $\beta$  was partially digested with 250 ng trypsin for 2 h at 25°C, reduced, carboxymethylated and injected onto a Vydac C18 Model 218TP54 4.6  $\times$  250 mm HPLC column and fractionated by a 0.5% min<sup>-1</sup> linear gradient of acetonitrile in 0.1% (v/v) trifluoroacetic acid at 0.7 ml min<sup>-1</sup>. Fractions (0.7 ml) were concentrated and analysed by sequencing. Residues identified were 121–123 and 125–126; 133–158, 162–167 and 169–171. *S. aureus* V8 protease-specific peptides were obtained as follows: 10.5  $\mu$ g (600 pmol) IL-1 $\beta$  was digested with 250 ng V8 protease for 8 h at 25°C, reduced, carboxymethylated and fractionated as described above. Residues identified were 181–186, 200–203, 213–218 and 230–238. Fragmentation by CNBr was performed on the same sample that had been used to obtain the N-terminal sequence. After 20 cycles of NH<sub>2</sub>-terminal sequencing, the filter in the glass block holder was removed and placed into a small beaker. The filter was saturated with 30  $\mu$ l of a solution containing 15 mg CNBr in 100  $\mu$ l 70% (v/v) HCOOH. The remaining solution was placed open in the beaker, the beaker sealed with Parafilm and placed in the dark. After 20 h at room temperature, the filter was dried under vacuum and placed back into the sequencer. Five simultaneous sequences were seen, differentiated from each other by their wide range of relative yields. We identified clearly residues 153–158; 161–167 and 169–171; 212–218, 220–224 and 225–226; 247–252; and 265–269. All these amino acids confirmed the sequence obtained from the cDNA.



b

|     |    |                                                                                 |      |
|-----|----|---------------------------------------------------------------------------------|------|
| -77 | 5' | TTGACGGACCAAGGCACACAGGCTGCTCTGGATTCTCTGACCAATCTTCATTGCTCAAGTGCTGAGGACGC         | -1   |
|     |    | ATG GCA GAA GTA CTT GAG CTC GCC AGT GAA ATG ATG CTT TAT TAC AGT GGC AAT GAG GAT | 60   |
|     |    | Met Ala Glu Val Pro Glu Leu Ala Ser Glu Met Met Ala Tyr Tyr Ser Gly Asn Glu Asp | 20   |
|     |    | GAC TTU TTC TTT GAA GCT GAT GGC CTT AAA CAG ATG AAG TCG TCC TTC CAG GAC CTU GAC | 120  |
|     |    | Asp Leu Phe Phe Glu Ala Asp Gly Pro Lys Gln Met Lys Cys Ser Phe Gln Asp Leu Asp | 40   |
|     |    | CTC TGC CTT CTG GAT GGC GGC ATC CAG TCA CGA ATC TCC GAC CAC CAC TAC ACC AAG GGC | 180  |
|     |    | Leu Cys Pro Leu Asp Gly Gly Ile Gln Leu Arg Ile Ser GAC His His Tyr Ser Lys Gly | 60   |
|     |    | TTC AGG CAG GCC GCG TCA GTT GTT GTG GCC ATG GAC AAG CTG AGG AAG ATG CTG GTT CCC | 240  |
|     |    | Phe Arg Gln Ala Ala Ser Val Val Val Ala Met Asp Lys Leu Arg Lys Met Leu Val Pro | 80   |
|     |    | TGC CCA CAG ACC TTC CAG GAG AAT GAC CTG AGC ACC TTC TTT CCC TTC ATC TTT GAA GAA | 300  |
|     |    | Cys Pro Gln Thr Phe Gln Glu Asn Asp Leu Ser Thr Phe Phe Phe Ile Phe Phe Glu Glu | 100  |
|     |    | GAA CTT ATC TTC TTT GAC ACA TGG GAT AAC GAG GCT TAT GTG CAC GAT GCA CTT GTA CCA | 360  |
|     |    | Glu Pro Ile Phe Phe Asp Thr Trp Asn Glu Ala Tyr Val His Asp Ala Pro Val Arg     | 120  |
|     |    | TCA CTG AAC TGC AGC CTC GCG GAC TCA CAG CAA AAA AGC TTG GTG ATG TCT GGT CCA TAT | 420  |
|     |    | Ser Leu Asn Cys Thr Leu Arg Asp Ser Gln Gln Lys Ser Leu Val Met Ser Gly Pro Tyr | 140  |
|     |    | GAA CTG AAA GCT CTC CAC CTC CAG GCA CAC GAT ATC CAG CAA CAA CTC CTC TTC TCC ATG | 480  |
|     |    | Glu Leu Lys Ala Leu His Leu Gln Gly Gln Asp Met Glu Gln Gln Val Val Phe Ser Met | 160  |
|     |    | TCC TTT GTA CAA GGA GAA GAA AGT AAT GAC AAA ATA CCT GTG GCC TTG GGC CTC AAG GAA | 540  |
|     |    | Ser Phe Val Gln Gly Glu Glu Ser Asn Asp Lys Ile Pro Val Ala Leu Gly Leu Lys Glu | 180  |
|     |    | AAG AAT CTG TAC CTG TCC TCC CTG TTG AAA GAT GAT AAG CCC ACT CTA CAG CTG GAG AGT | 600  |
|     |    | Lys Asn Leu Tyr Leu Ser Cys Val Leu Lys Asp Asp Lys Pro Thr Leu Gln Leu Glu Ser | 200  |
|     |    | GTA GAT CCC AAA AAT TAC CCA AAG AAG AAG ATG GAA AAG CGA TTT GTC TTC AAC AAG ATA | 660  |
|     |    | Val Asp Pro Lys Asn Tyr Pro Lys Lys Lys Met Glu Lys Arg Phe Val Phe Asn Lys Ile | 220  |
|     |    | GAA ATC AAT AAC AAG CTC GAA TTT GAG TCT CCC CAG TTC CCC AAC TGG TAC ATC ACC ACC | 720  |
|     |    | Glu Ile Asn Asn Lys Leu Glu Phe Glu Ser Ala Gln Phe Pro Asn Trp Tyr Ile Ser Thr | 240  |
|     |    | TCT CAA GCA GAA AAC ATG CCC GTC TTC CTC GGA GGC ACC AAA GGC GGC CAG GAT ATA ACT | 780  |
|     |    | Ser Gln Ala Glu Asn Met Pro Val Phe Leu Gly Gly Thr Lys Gly Gly Gln Asp Ile Thr | 260  |
|     |    | GAC TTC ACC ATG CAA TTT GTG TCT TCC TAA AGAGAGCTGTACCCAGAGAGCTCTCTGCTCAATGTCAC  | 849  |
|     |    | Asp Phe Thr Met Gln Phe Val Ser Ser End                                         | 269  |
|     |    | TCAATCCCTAGGGCTGGCAAGGGAACAGAAAGTTTGTAGTACGGCTATACGCTGGACITTCCTGTGTCTACAC       | 928  |
|     |    | CATGCGCAACTGCTGCTTAGGGTAGTCTAAGAGAGATCTCTGTCATCAGCCAGGACAGTCAAGCTCTCTCTTC       | 1007 |
|     |    | AGGGCCATCCAGCCCTTTTGTGTAGCCAGGCTCTCT -3' 1047                                   |      |

Nucleotide sequence analysis of p2C1 showed that it contained a non-coding region of IL-1 mRNA. Therefore, nick-translated and oligonucleotide probes derived from p2C1 were used to isolate further homologous cDNA clones from the library (Fig. 1). 5' probes from these new clones were then used to identify further cDNA clones, each able to select biologically active IL-1 mRNA (data not shown). The nucleotide sequence of IL-1 mRNA defined by the overlapping cDNA clones is shown in Fig. 1. It predicts an mRNA size of at least 2,002 nucleotides, excluding the poly(A) tail. Primer extension analysis indicated that the IL-1 mRNA extends ~15 nucleotides upstream of the sequence shown here (data not shown). A single, large open reading frame of 271 amino acids was found at the 5' end of the mRNA encoding a protein of predicted *M*<sub>r</sub> 30,606 (designated IL-1 $\alpha$ ). Although IL-1 is thought to be a secreted protein, the predicted sequence does not contain a classical N-terminal signal sequence of largely hydrophobic amino acids such as are found in other lymphokines<sup>14–17</sup>, nor does it have any long internal hydrophobic amino-acid sequences.

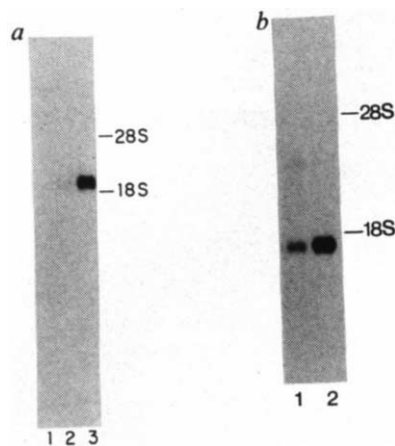
While this work was in progress, Lomedico *et al.* published the cloning and sequencing of a murine IL-1 cDNA<sup>10</sup>. Comparison of the two predicted amino-acid sequences reveals that they are 62% homologous, suggesting that the gene cloned here is the human homologue. They have also defined the N-terminus of a subset of biologically active murine IL-1 molecules at

Ser 115 (ref. 10). By matching the sequences of murine IL-1 and human IL-1 $\alpha$ , this corresponds to Ser 113 in the human sequence. The relationship between different IL-1 molecules will be discussed below.

## Cloning of IL-1 $\beta$

In a separate approach to the definition of IL-1, we have reported previously the purification (from activated macrophage supernatants) of a *M*<sub>r</sub> 17,500 protein with IL-1 activity<sup>2</sup>. We attempted to sequence this molecule to design oligonucleotide probes to identify IL-1 cDNA clones. However, amino-terminal sequence analysis of this purified protein yielded ambiguous results because of a low absolute yield in the first cycle, followed by a very sharp decline in repetitive yield. Therefore, we fragmented the molecule enzymatically. We performed a partial tryptic digest on 600 pmol protein and, after reduction and carboxymethylation, fractionated the resulting peptides by reversed-phase HPLC. A *M*<sub>r</sub> 13,500 peptide was isolated in high yield. Sequence analysis of this fragment showed a continuous stretch of 26 amino acids followed by 10 identifiable residues in the subsequent 14 cycles. The 26 amino-acid stretch (Ser 133–Phe 158; Fig. 2) was used to design (based on published human codon frequencies) a synthetic, 62-base oligonucleotide probe (see Fig. 2 legend) which was <sup>32</sup>P-labelled and used to screen the size-fractionated macrophage cDNA library. Several positively





**Fig. 3** Northern blot analysis of: *a*, IL-1 $\alpha$ ; *b*, IL-1 $\beta$  mRNA. *a*, Lane 1, 5  $\mu$ g total RNA from mitogen-stimulated peripheral blood T cells; lane 2, unstimulated peripheral blood macrophages; lane 3, LPS-stimulated peripheral blood macrophages. *b*, Lane 1, 5  $\mu$ g total RNA from unstimulated peripheral blood macrophages; lane 2, LPS-stimulated peripheral blood macrophages.

**Methods.** Total RNA was extracted from LPS-stimulated and unstimulated macrophages as in Fig. 1, and from peripheral blood T cells stimulated with the mitogens concanavalin A (20  $\mu$ g ml<sup>-1</sup>) and phorbol myristate acetate (10 ng ml<sup>-1</sup>) as described previously<sup>35</sup>. RNA was electrophoresed on 1.5% agarose gels containing formaldehyde and transferred to nitrocellulose filters<sup>12</sup>. Blots were hybridized with <sup>32</sup>P-RNA probes synthesized by SP6 polymerase as described previously<sup>42</sup>. The IL-1 $\alpha$  probe used was derived by subcloning a 1,020-bp *Pst*I/*Hind*III fragment from p9H into pSP64 (Promega Biotec). The IL-1 $\beta$  probe was derived by subcloning the insert from pIL-1-14 into the *Pst*I site of pSP64. Hybridization was for 16 h at 63 °C in Stark's buffer<sup>43</sup>. Blots were washed in 0.1  $\times$  SSC for 30 min at 68 °C and autoradiographed with intensifying screens. The position of large (28S) and small (18S) ribosomal RNAs indicated were determined by direct visualization of the unbaked blot with a short-wave ultraviolet lamp.

hybridizing clones were isolated, one of which, pIL-1-14, was chosen for nucleotide sequencing (Fig. 2). The clone, 1,124 base pairs (bp) long, contained the probe sequence with 12 mismatches out of 62 nucleotides and encoded the protein sequence obtained from the *M<sub>r</sub>* 13,500 tryptic fragment.

The sequence of pIL-1-14 defined a single large open reading frame of 269 amino acids encoding a protein with a predicted *M<sub>r</sub>* of 30,749 (Fig. 2). We found a presumptive initiator methionine codon flanked by sequences closely conforming to the consensus sequence suggested for mammalian translational initiation<sup>18</sup> 78 bp from the 5' end of the pIL-1-14 cDNA sequence. The cDNA insert contained 240 nucleotides of 3' non-coding sequence but did not extend far enough to include the polyadenylation site and poly(A) tail. Primer extension analysis indicated that the message extended ~10 nucleotides 5' of pIL-1-14 (data not shown). The predicted amino-acid sequence of the protein encoded by pIL-1-14 (designated IL-1 $\beta$ ) showed only limited homology to that of IL-1 $\alpha$  (see below).

To establish the location of the *M<sub>r</sub>* 17,500 form of IL-1 in the 269 amino-acid open reading frame, we sequenced a larger preparation (~680 pmol) of purified IL-1 $\beta$ , and could distinguish a major signal corresponding to the sequence starting at Ala 117, and which agreed with the sequence predicted from pIL-1-14 for the subsequent 20 amino acids (Fig. 2). Although a yield of only 266 pmol (39%) was obtained for the N-terminal sequencing run, no other discrete N-termini were found. These results were confirmed by additional runs on the same, and other, preparations of IL-1 protein. A similar result was reported for murine IL-1 (ref. 10). Sequence analysis of peptides generated from a *Staphylococcus aureus* V8 protease digest and from *in situ* cyanogen bromide cleavage of IL-1 $\beta$  (Fig. 2 legend) yielded additional confirmation of protein sequence predicted by the cDNA sequence at 96 positions of the 153 amino-acid

sequence (capitalized amino acids in Fig. 2) predicted for the *M<sub>r</sub>* 17,500 IL-1 $\beta$ , including definition of the carboxy-terminus at Ser 269. A potential *N*-glycosylation site is present at Asn 123, although there is no previous evidence that IL-1 $\beta$  is a glycoprotein<sup>2</sup>. Direct protein sequencing detected asparagine at this position, confirming the absence of carbohydrate. Thus, the *M<sub>r</sub>* 17,500 form of IL-1 $\beta$  extends from Ala 117 to Ser 269 and represents a carboxy-terminal segment of the primary translation product. The predicted *M<sub>r</sub>* of this form of IL-1 $\beta$  is 17,377, in close agreement with the *M<sub>r</sub>* of 17,500 determined previously<sup>2</sup>.

Similar to IL-1 $\alpha$ , the amino-acid sequence predicted for the primary translation product of IL-1 $\beta$  does not contain any N-terminal or internal stretches of hydrophobic amino acids that resemble conventional secretory signal sequences.

## IL-1 mRNA expression

We analysed IL-1 mRNA levels in stimulated and unstimulated macrophages by Northern blotting using IL-1 $\alpha$  and IL-1 $\beta$ -specific probes (Fig. 3). In both cases, we detected a single predominant species of RNA much more abundant in LPS-stimulated than unstimulated macrophage RNA. The low levels of IL-1 RNA in unstimulated macrophages resulted from partial activation during culture as cells cultured under pyrogen-free conditions do not have detectable IL-1 RNA (K.G. and A.L., unpublished). The sizes were estimated to be 2,100–2,200 nucleotides for IL-1 $\alpha$  mRNA and ~1,800 for IL-1 $\beta$ . Neither IL-1 $\alpha$  mRNA (Fig. 3*a*) nor IL-1 $\beta$  mRNA (data not shown) was detectable in RNA extracted from peripheral blood T cells activated with the mitogens concanavalin A and phorbol myristate acetate, even on longer exposure of the autoradiogram.

Interestingly, IL-1 $\beta$  mRNA seemed to be ~10-fold more abundant than IL-1 $\alpha$  mRNA. IL-1 $\alpha$  cDNA clones were present at a frequency of ~1 in 1,000 in the size-fractionated cDNA library, whereas IL-1 $\beta$  cDNA clones were present at ~1 in 100. Given an estimated enrichment for IL-1 mRNA by agarose gel electrophoresis of ~10-fold, this would predict abundances of ~0.01% and 0.1% of the poly(A)<sup>+</sup> RNA from LPS-stimulated macrophages for IL-1 $\alpha$  and IL-1 $\beta$  mRNAs, respectively.

Although IL-1 $\alpha$  and IL-1 $\beta$  mRNAs are induced by the same stimulus (LPS), it is not known whether they are produced by the same cell type in the peripheral blood monocyte population. The availability of specific nucleic acid and antibody probes for IL-1 $\alpha$  and IL-1 $\beta$  should clarify this issue.

## Expression of IL-1 $\alpha$ and IL-1 $\beta$ activity

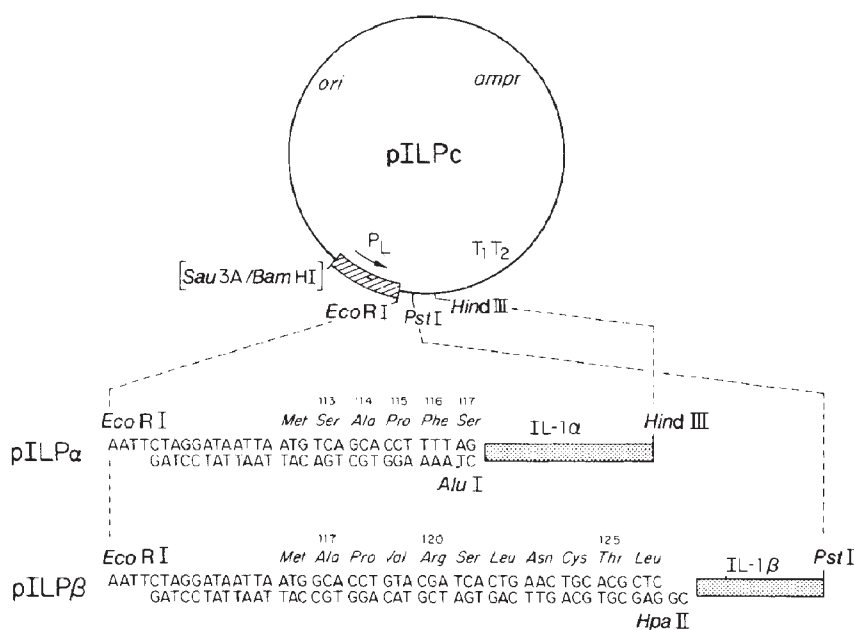
Evidence presented here for IL-1 $\beta$ , and by Lomedico *et al.* for the murine homologue of IL-1 $\alpha$ <sup>10</sup>, suggests that the biological activity of the two IL-1 molecules resides in their C-terminal 153 and 159 amino acids, respectively. To prove this, we constructed plasmids designed to direct synthesis by *E. coli* of these portions of IL-1 $\alpha$  and IL-1 $\beta$ . We used synthetic oligonucleotides that added a ribosome binding site and initiation codon to the C-terminal 159 amino acids of IL-1 $\alpha$  (beginning at Ser 113) or to the C-terminal 153 amino acids of IL-1 $\beta$  (beginning at

**Table 2** Expression of IL-1 biological activity

| Plasmid       | IL-1 in culture (U ml <sup>-1</sup> ) |                              |
|---------------|---------------------------------------|------------------------------|
|               | 30 °C                                 | 43 °C                        |
| pILP $\alpha$ | 3.2 $\times$ 10 <sup>3</sup>          | 5.1 $\times$ 10 <sup>6</sup> |
| pILP $\beta$  | 4.1 $\times$ 10 <sup>2</sup>          | 1.4 $\times$ 10 <sup>6</sup> |
| pILPc         | ND                                    | ND                           |

Cells were grown at 30 °C to an *A*<sub>720</sub> = 1.0 in induction broth (M9 medium supplemented with 32 g l<sup>-1</sup> tryptone, 20 g l<sup>-1</sup> yeast extract and 0.4% glucose). Cultures were then grown at 43 °C for 1 h. Cells from 1 ml culture were pelleted and quick-frozen on dry ice/methanol. Before assay the cells were dissolved in 50  $\mu$ l 7 M guanidine hydrochloride. IL-1 biological activity was assayed as described for Fig. 1*a*. Results are expressed as IL-1 units per ml *E. coli* culture. ND, not detected. No biological activity over background control at a sample dilution of 1:50 (IA5 assay).

**Fig. 4** The expression plasmid, pILPc, was constructed by replacing the *Bam*HI/*Eco*RI fragment of pKK223-3 (Pharmacia), containing the *Tac* promoter, with a *Sau*3A/*Eco*RI fragment from pPLc28 (ref. 44) that contains the  $\lambda$   $P_L$  promoter. Synthetic oligonucleotides were used to provide an *E. coli* ribosome binding site, a translational initiation codon and the codons for amino acids Ser 113–Ser 117 for IL-1 $\alpha$  or Ala 117–Leu 126 for IL-1 $\beta$ . To construct pILP $\alpha$ , the oligonucleotides were ligated together with a 720-bp pair *Alu*I/*Hind*III fragment into *Eco*RI/*Hind*III-cut pILPc. This fragment was derived from the yeast expression plasmid, pY $\alpha$ IL-1 $\alpha$  (V.P., unpublished), and specifies amino acids 118–271 of IL-1 $\alpha$  followed by 200 bp of sequence originally derived from the 3' non-coding region of the human *IL-2* gene. To construct pILP $\beta$ , the oligonucleotides were ligated together with a 669-bp *Hpa*II/*Pst*I fragment from pIL-1-14 into *Eco*RI/*Pst*I-cut pILPc. This fragment specifies amino acids 127–269 of IL-1 $\beta$ .  $T_1T_2$ , transcriptional termination sites from the *rrnB* operon pILPc, pILP $\alpha$  and pILP $\beta$  transformed into *E. coli* strain  $\Delta$ HI (ref. 45) that produces a thermolabile repressor of the  $P_L$  promoter.



Ala 117), and placed this under transcriptional control of the  $\lambda$  phage  $P_L$  promoter (Fig. 4). The resulting plasmids, pILP $\alpha$  and pILP $\beta$ , were introduced into cells containing a thermolabile repressor of  $P_L$  transcription. On heat induction, the cells produced high levels of IL-1 activity that could be extracted from the cells by 7 M guanidine hydrochloride as detected in the 1A5 conversion assay (Fig. 4, Table 2) or thymocyte co-mitogenesis assay<sup>2</sup> (data not shown). No IL-1 activity was found in cells transformed with a control plasmid lacking IL-1 sequences.

### Interleukin-1 relationships

To determine the degree of homology between the IL-1 $\beta$  sequence and the related sequences for human IL-1 $\alpha$  and murine IL-1<sup>10</sup>, we aligned them as shown in Fig. 5a, with several insertion/deletion gaps to obtain the maximum number of identical residues. The amino-acid sequences for human IL-1 $\alpha$  and murine IL-1 are closely related, having 167 out of 271 (62%) identical positions, whereas IL-1 $\beta$  is only distantly related to either of the other molecules, with 70 out of 271 identical positions (26%) with human IL-1 $\alpha$  and 80 out of 270 (30%) with murine IL-1. Slightly over half of the identical positions between the IL-1 proteins occur in the  $M_r$  17,500, C-terminal biologically active portion of the molecules, and a large proportion of these occur in the middle and C-terminal regions (Val 164–Ile 266 of IL-1 $\alpha$ ) of the  $M_r$  17,500 forms, suggesting that these areas comprise portions of the molecules more critical for their biological activities. In contrast, very few residues are shared between the three proteins in the N-terminal third (Ser 113–Ala 163 of IL-1 $\alpha$ ) of the processed IL-1 sequences. This sequence diversity suggests that the N-terminal region of the  $M_r$  17,500 form either is not involved in a biological role or provides an area for expression of different biological functions in each molecule.

Of the residues common to all three proteins, 47% (28 of 59) occur in the portion of the primary translation product that precedes the active  $M_r$  17,500 IL-1 segment. In this region, 81 out of the 167 identical residues between human IL-1 $\alpha$  and murine IL-1 occur, showing that these portions of the molecules are more homologous (81 of 112; 72%) than the  $M_r$  17,500 IL-1 segments (86 of 159; 54%). IL-1 $\beta$  also has more homology with both molecules in this region. Such a degree of homology would not be expected for a non-functional portion of a protein. Therefore, it seems plausible that the N-terminal halves of the IL-1 gene products serve some as yet unknown biological func-

tion. Indeed, several biological activities have been associated with high  $M_r$  forms of IL-1<sup>8,19</sup>, some of which may be related to the conserved N-terminal segment.

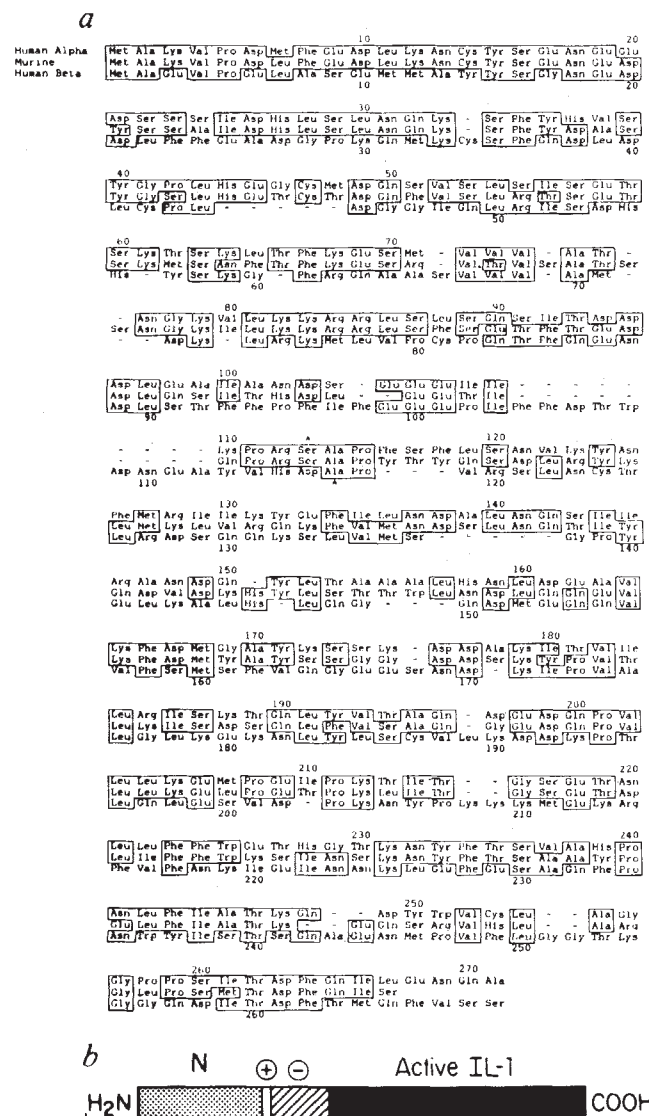
Note that although there is only 26% homology between the two human IL-1 protein sequences, significantly more occurs at the nucleic acid level (45%). Thus, it is possible that a gene duplication event gave rise to the two IL-1 molecules. The degree of divergence between the two sequences indicates that the duplication was an early event on the evolutionary scale<sup>20</sup>.

The structural features common to all three IL-1 polypeptides are illustrated in schematic form in Fig. 5b. All three primary translation products have a higher degree of homology in the amino-terminal 75–80 residues. This segment (N in Fig. 5b) does not seem to contain a signal sequence or membrane spanning region. In fact, it has no features that would serve to distinguish it from typical globular protein sequences. Following the N-terminal domain, all three IL-1 molecules have a short section of multiple basic residues (+ in Fig. 5b). This segment is reminiscent of known proteolytic processing sites in pro-hormone precursors<sup>21,22</sup>, maturation cleavage sites of the haemagglutinin protein of influenza virus<sup>23</sup> and complement component C3 (ref. 24). Although the mechanism for IL-1 processing is unknown, it is tempting to speculate that this region serves as a proteolytic processing site to yield an intermediate polypeptide from which biologically active fragments are produced by further proteolytic cleavage. The segment following the multiple basic residues is less conserved in the three molecules, but in all cases, this segment (– in Fig. 5b) contains many acidic amino acids. No basic residues are found in this region, except just before the amino terminus of the active IL-1 segments. Such a negatively charged region, present in all three molecules, probably has a conserved structural or functional role. The rest of the sequence comprises the processed active  $M_r$  17,500 IL-1 region.

### Discussion

We have isolated cDNA clones representing two distinct but distantly related forms of human IL-1, IL-1 $\alpha$  and IL-1 $\beta$ , from LPS-stimulated macrophage RNA. Although the nucleotide sequence of the cDNA clones predicts primary translation products of  $M_r$  30,606 and 30,749 for IL-1 $\alpha$  and IL-1 $\beta$ , respectively, we have shown that the C-terminal 159 and 153 amino acids are sufficient for the production of IL-1 biological activity. Previous reports have characterized IL-1 activities as having





**Fig. 5** *a*, Alignment of human IL-1 $\alpha$ , murine IL-1 $\beta$  and human IL-1 $\beta$  amino-acid sequences. Numbering begins at the presumed initiator methionine; numbering above the top row is for IL-1 $\alpha$ ; that below the bottom row is for IL-1 $\beta$ . Boxed residues indicate identities between two or more of the sequences. Star above Ser 113, presumed NH<sub>2</sub>-terminus of IL-1 $\alpha$ ; star below Ala 117 of IL-1 $\beta$  NH<sub>2</sub>-terminus as defined by protein sequencing. Alignment presented was determined by a combination of computerized methods<sup>20,46</sup>, hydrophilicity analysis<sup>47</sup> and visual inspection. *b*, Schematic representation of the common features of IL-1 polypeptides. Stippled box (N), N-terminal conserved region from Met 1 to Gly 78 in IL-1 $\alpha$ , or to Asp 72 in IL-1 $\beta$ . Small open box (+), multiple basic region (Lys 79-Arg 85 in IL-1 $\alpha$ , or Lys 73-Lys 76 in IL-1 $\beta$ ). Hatched box (-), negatively charged region (Leu 86-Arg 112 in IL-1 $\alpha$ , or Met 77-Asp 116 in IL-1 $\beta$ ). Closed box ('Active IL-1'), C-terminal region of the primary translation product representing the extent of the processed, active M<sub>r</sub> 17,500 IL-1 (Ser 113-Ala 271 for IL-1 $\alpha$ ; Ala 117-Ser 269 for IL-1 $\beta$ ).

M<sub>r</sub>s of ~30,000 and 17,000. Our results resolve this inconsistency and support the concept that human IL-1s are synthesized as higher M<sub>r</sub> precursors that are proteolytically cleaved to M<sub>r</sub> 17,500 active forms, as has been proposed for murine IL-1<sup>8</sup>. However, we have not yet defined all of the naturally occurring, biologically active forms of IL-1 $\alpha$  and IL-1 $\beta$ ; this will require the generation of specific antisera against these molecules.

An interesting difference between IL-1 $\alpha$  and IL-1 $\beta$  is that the primary translation product of IL-1 $\alpha$  seems to be biologically active, judged by the production of IL-1 activity by hybrid-selected IL-1 $\alpha$  mRNA in a rabbit reticulocyte lysate (Table 1). In contrast, hybrid-selected IL-1 $\beta$  mRNA was not biologically

active under the same conditions, even though a protein corresponding to the IL-1 $\beta$  primary translation product could be seen clearly when the hybrid-selected IL-1 $\beta$  mRNA was translated in a reticulocyte lysate supplemented with <sup>35</sup>S-methionine and analysed on an SDS-polyacrylamide gel (data not shown). It is also possible that the primary translation product of IL-1 $\alpha$  is more efficiently cleaved to a smaller active form. This suggestion is supported by the presence of a basic amino acid immediately before Ser 113 that might be a cleavage site for a trypsin-like protease.

Unlike other secreted proteins, IL-1 $\alpha$  and IL-1 $\beta$  do not contain a stretch of hydrophobic amino acids, either at the N-terminus or internally, raising the possibility that IL-1 is not actively secreted by macrophages, but is released by damaged cells<sup>25</sup>. This conclusion is supported by the inefficient release of IL-1 activity by *Xenopus* oocytes injected with IL-1 mRNA or by COS-7 monkey kidney cells transfected with IL-1 expression vectors (data not shown). Alternatively, macrophages may export IL-1 by a mechanism distinct from that described for other secretory proteins.

Recently, Auron *et al.* reported the sequence of a cDNA clone that is very similar to IL-1 $\beta$ <sup>11</sup>. There are seven nucleotide substitutions between the two sequences, five of which are silent changes. Plasmid pIL-1-14 has an adenine instead of a guanine at nucleotide 16, changing lysine to glutamic acid, and lacks the upstream, out of frame, potential initiation codon found in the sequence of Auron *et al.* (position -23 in Fig. 2). All the nucleotide substitutions in our sequences have been verified by sequencing other IL-1 $\beta$  cDNA clones, and presumably represent allelic differences or reverse transcriptase errors. Van Damme *et al.* have recently described the N-terminal amino acid sequence of a secreted factor with strong homology to the predicted amino acid sequence of the central region of the IL-1 $\beta$  cDNA clone<sup>48</sup>.

It is striking that two distinct molecules should mediate the induction of IL-2 synthesis. Whether they share all the biological activities previously attributed to IL-1 is under investigation. It will now be possible to determine if both IL-1 $\alpha$  and IL-1 $\beta$  bind to the same receptor, analogous to the binding of the structurally distinct epidermal growth factor (EGF) and transforming growth factor- $\alpha$  molecules to the EGF receptor<sup>26</sup>. Resolution of these issues should increase our understanding of the biological role of IL-1 in immunity and inflammation.

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## LETTERS TO NATURE

### Observation of highly excited radio recombination lines towards Cassiopeia A

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The absorption line at 26 MHz observed by Konovalenko and Sodin<sup>1</sup> towards Cas A was interpreted by Blake *et al.*<sup>2</sup> as the 631 $\alpha$  recombination line due to carbon or heavier elements. This was confirmed subsequently by Konovalenko and Sodin<sup>3</sup>, who detected the 640 $\alpha$  line at the same optical depth as the 631 $\alpha$  line, within error limits. These lines represent the highest excited states yet detected in interstellar gas and could provide<sup>4</sup> a unique diagnostic of conditions in diffuse clouds if observed over a range of frequencies. We now report new observations of these lines carried out near 26, 38, 52 and 68 MHz using the National Radio Astronomy Observatory (NRAO) 91-m telescope at Green Bank. We detected absorption lines at all these frequencies, with a striking fourfold increase in width towards low frequencies due to collisional or radiation broadening. Assuming that these are recombination lines of carbon, the observed radial velocity corresponds well with that of the H I, OH, NH<sub>3</sub> and H<sub>2</sub>CO absorption features that originate in the Perseus arm in the same direction<sup>5-8</sup>.

The 68- and 52-MHz observations were made in July 1984, using the 50-80-MHz tunable dipole on the travelling feed system. The 38- and 25-MHz observations were made in August 1984 using a broadband 20-50-MHz dipole especially made for this experiment and mounted on the travelling feed, which is capable of tracking a source for 76 sec  $\delta$  minutes, where  $\delta$  is the declination. In the frequency range of these observations, the system temperature was dominated by Cas A and the galactic background. At each of the four frequencies, two or three nearby transitions in carefully chosen interference-free bands were observed simultaneously. The 384-channel autocorrelation receiver was split into four independent spectrometers of 96 channels each, providing a frequency resolution of 0.98 kHz at the two higher and 0.49 kHz at the two lower frequencies. Reference spectra were measured by switching to a noise source which was adjusted to match the total system temperature when the antenna was pointed to Cas A.

Table 1 Observed line parameters

| Frequency (MHz) | $V_{\text{LSR}}$ (km s <sup>-1</sup> ) | $T_L/T_C$ ( $\times 10^3$ ) | $\Delta V$ (km s <sup>-1</sup> ) | $\int \tau d\nu$ (s <sup>-1</sup> ) |
|-----------------|----------------------------------------|-----------------------------|----------------------------------|-------------------------------------|
| 26              | $-45 \pm 5$                            | $-3.8 \pm 0.5$              | $32.9 \pm 2.1$                   | $11.6 \pm 0.7$                      |
| 38              | $-49 \pm 2$                            | $-3.5 \pm 0.4$              | $17.9 \pm 0.9$                   | $8.4 \pm 0.5$                       |
| 52              | $-49 \pm 2$                            | $-3.5 \pm 0.4$              | $13.0 \pm 0.7$                   | $8.6 \pm 0.5$                       |
| 68              | $-48 \pm 2$                            | $-2.9 \pm 0.6$              | $8.3 \pm 0.9$                    | $5.9 \pm 0.8$                       |

Errors quoted are 1 $\sigma$  values.  $V_{\text{LSR}}$  is the radial velocity with respect to the local standard of rest;  $T_L$  and  $T_C$  are the line and continuum antenna temperatures respectively;  $\Delta V$  is the full linewidth at half maximum intensity;  $\int \tau d\nu$  is the optical depth integrated over the line.

The spectra obtained after removing a linear baseline at each of the four frequencies are shown in Fig. 1. The recombination line is seen in absorption at all the four frequencies at the velocity corresponding to the Perseus arm. In the spectra in Fig. 1 there are suggestions, at least at the three lower frequencies, that the absorption line may be present at the velocities of both  $-47$  and  $-39$  km s<sup>-1</sup> at which H I absorption is seen in the Perseus arm<sup>5</sup>. However, we find no evidence in our spectra of a line at 0 km s<sup>-1</sup> corresponding to the Orion arm absorption, and no hydrogen recombination lines. The line parameters obtained by fitting a single gaussian to each of the observed spectra are given in Table 1. As Cas A dominates the system temperature at these frequencies, the line-to-continuum ratio plotted in Fig. 1 and given in Table 1 is approximately equal to the observed optical depth.

All high-frequency observations (mainly towards H II regions) show recombination lines of hydrogen or heavier elements only in emission. However, there are good reasons for expecting recombination lines to be seen in absorption at very low frequencies particularly from cold gas<sup>2</sup> and in the presence of strong background radiation. At very large  $n$  (the principal quantum number) electron collisions dominate (as among free electrons) and therefore maintain thermalization of the populations. It is only at lower  $n$  (and correspondingly higher frequencies) that radiative processes begin to dominate and the excitation temperature moves away from the kinetic temperature of

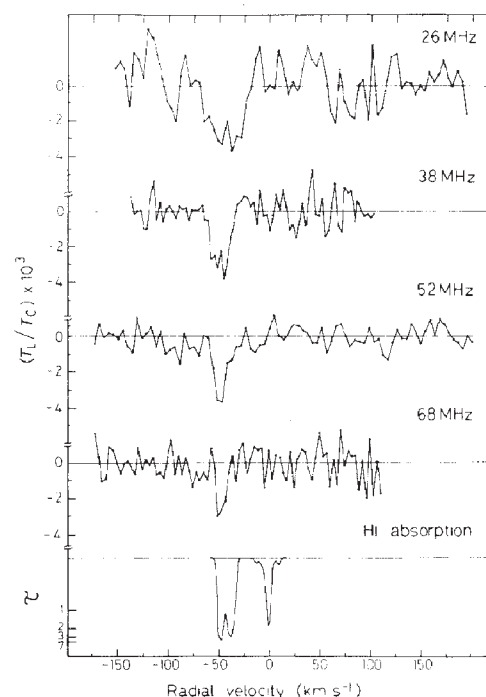
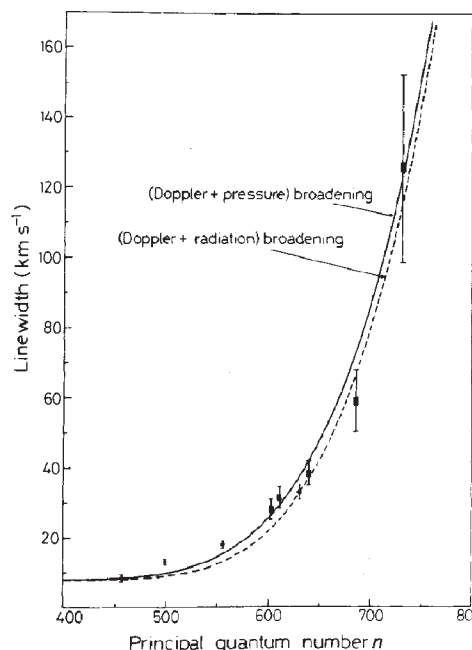
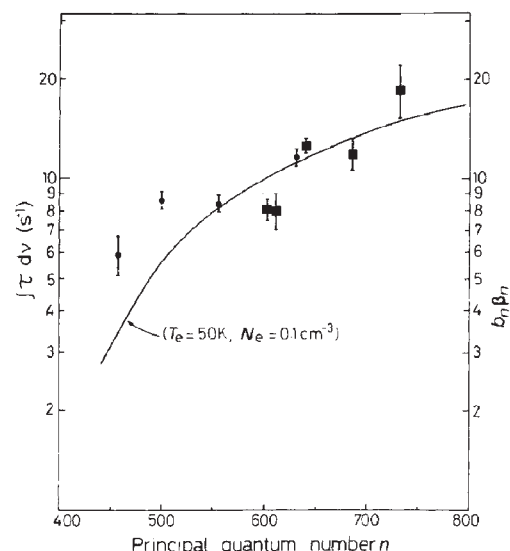


Fig. 1 Recombination line spectra towards Cas A observed at four frequencies. At each frequency the spectrum is an average of two or three nearby transitions that were observed simultaneously. These transitions are 26 MHz, 629 $\alpha$  and 634 $\alpha$ ; 38 MHz, 554 $\alpha$  and 556 $\alpha$ ; 52 MHz, 497 $\alpha$ , 501 $\alpha$  and 503 $\alpha$ ; 68 MHz, 456 $\alpha$ , 457 $\alpha$  and 458 $\alpha$ . The effective integration times for the four frequencies are 4.4, 10.7, 10.5 and 9.6 h respectively. The H I absorption spectrum is from ref. 5.





**Fig. 2** Calculated and observed variation of line width (FWHM). ●, Present observations; ■, data from ref. 12. Pressure broadening is calculated for  $T_e = 50$  K and  $N_e = 0.18 \text{ cm}^{-3}$  and for radiation broadening a brightness temperature  $T_R = 2,000$  K at 100 MHz ( $\alpha = -2.6$ ) was used. A Doppler component of  $8 \text{ km s}^{-1}$  is assumed for both cases. The relevant formulas are taken from ref. 13.



**Fig. 3** The observed variation of integrated line intensity. The quantity  $b_n \beta_n$  (solid curve) taken from ref. 9 is normalized to a value of 10 at  $n = 600$ . This quantity which is proportional to the net optical depth was calculated by Walmsley and Watson<sup>9</sup> based on a dielectronic recombination process. ●, Present observations; ■, data from ref. 12.

the gas, towards negative values, so that the line can never be seen in absorption irrespective of the background radiation. The turnover frequency will, however, depend on the density and temperature of the gas and the populating mechanism<sup>9</sup> for the species of atom in question.

As pointed out by Blake *et al.*<sup>2</sup>, the identification of the heavy element responsible for the line towards Cas A cannot be made on the basis of frequency alone. It is most likely to be carbon if it originates in cold gas. Alternatively, it could be due to heavier elements such as calcium or magnesium if located in hot gas<sup>2,10,11</sup>. In the present case, however, the agreement in velocity with the H I and molecular absorption features strongly favours cold dense clouds as the site of absorption, and carbon as the element in question.

The observed width of the line at 68 and 52 MHz is similar to that of the H I absorption line. The width, in velocity units, increases by nearly a factor of four at 26 MHz. This line has now been observed down to 16.7 MHz by Konovalenko<sup>12</sup> and the width increases even more dramatically (Fig. 2), which clearly indicates pressure and/or radiation broadening. Using the formula given by Shaver<sup>13</sup> for the collisional width at low temperatures we infer an upper limit of  $0.2 \text{ cm}^{-3}$  for the electron density of the gas responsible for the observed lines, assuming a temperature of 50 K. If the carbon in this cloud has a cosmic abundance ( $C/H = 3 \times 10^{-4}$ ) and all of it is ionized, then this implies an upper limit to the hydrogen density of  $600 \text{ cm}^{-3}$ . This is consistent with the upper limit to the H I density obtained from the measured column density and the minimum size of the cloud implied by its high H I optical depth. From consideration of radiation broadening<sup>13</sup>, we infer an upper limit of 2,000 K (at 100 MHz) for the ambient brightness temperature for the recombination line gas (see Fig. 2). This further implies a lower limit of 150 pc for the distance of the absorbing gas from Cas A, if we take the flux of Cas A at 100 MHz to be  $15,000 \text{ Jy}$  (ref. 14) and its distance and linear size to be 4 kpc and 5 pc, respectively.

At temperatures of 50–100 K, which are typical for H I clouds, calculations by Walmsley and Watson<sup>4,9</sup> show that dielectronic-like recombination on to carbon could be an important populating mechanism. This predicts an enhanced absorption at low

frequencies and a variation of line strength as a function of frequency which depends on the electron density in the gas. At low electron densities ( $N_e \sim 0.01 \text{ cm}^{-3}$ ) a steep decrease in line strength from 25 to 100 MHz is predicted. From Table 1, although the observed peak optical depth in the line remains nearly constant in the frequency range 68–26 MHz, the integrated absorption ( $\int \tau dv$ ) increases roughly by a factor of 2. If the dielectronic recombination process is operating, then the observed variation of the total line strength indicates an electron density of  $\sim 0.1 \text{ cm}^{-3}$  (see Fig. 3) and rules out much lower densities.

Alternatively, these lines may be formed by carbon associated with dense molecular clouds rather than by carbon associated with more widely distributed H I. Batrla *et al.*<sup>7</sup> have observed two such clouds in  $\text{H}_2\text{CO}$  absorption against Cas A, each with a diameter of about 1 arc min and with velocities of  $-39$  and  $-47 \text{ km s}^{-1}$  respectively. These are foreground clouds in the Perseus arm which cover only a few per cent of the solid angle subtended by Cas A. If the low-frequency absorption lines are formed in the molecular clouds, then the true optical depths of these lines would be 20–30 times higher than the average optical depths that we observe.

It must be possible to observe these highly excited lines in many other directions in the galactic plane. At these low frequencies, the non-thermal galactic radiation can provide the background continuum and the presence of a discrete background source may not be necessary for the detection of the lines. The present observations have indicated that recombination lines below 100 MHz will be an important diagnostic of the interstellar medium as was emphasized by Shaver<sup>13</sup>.

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## Analytical solution for the effect of increasing CO<sub>2</sub> on global mean temperature

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Increasing atmospheric carbon dioxide concentration is expected to cause substantial changes in climate<sup>1</sup>. Recent model studies suggest that the equilibrium warming for a CO<sub>2</sub> doubling ( $\Delta T_{2\times}$ ) is about 3–4 °C<sup>2–4</sup>. Observational data show that the globe has warmed by about 0.5 °C over the past 100 years<sup>5,6</sup>. Are these two results compatible? To answer this question due account must be taken of oceanic thermal inertia effects, which can significantly slow the response of the climate system to external forcing. The main controlling parameters are the effective diffusivity of the ocean below the upper mixed layer ( $\kappa$ ) and the climate sensitivity (defined by  $\Delta T_{2\times}$ ). Previous analyses of this problem have considered only limited ranges of these parameters. Here we present a more general analysis of two cases, forcing by a step function change in CO<sub>2</sub> concentration and by a steady CO<sub>2</sub> increase. The former case may be characterized by a response time which we show is strongly dependent on both  $\kappa$  and  $\Delta T_{2\times}$ . In the latter case the damped response means that, at any given time, the climate system may be quite far removed from its equilibrium with the prevailing CO<sub>2</sub> level. In earlier work this equilibrium has been expressed as a lag time, but we show this to be misleading because of the sensitivity of the lag to the history of past CO<sub>2</sub> variations. Since both the lag and the degree of disequilibrium are strongly dependent on  $\kappa$  and  $\Delta T_{2\times}$ , and because of uncertainties in the pre-industrial CO<sub>2</sub> level, the observed global warming over the past 100 years can be shown to be compatible with a wide range of CO<sub>2</sub>-doubling temperature changes.

We use an energy balance climate model consisting of two atmospheric boxes, one over land with zero heat capacity, and the other over an oceanic mixed layer coupled to a diffusive deeper ocean (compare refs 7–9). On timescales relevant to CO<sub>2</sub> forcing, the atmosphere may be assumed to be in equilibrium with the underlying surface. The model is then described by

$$\Delta T_L = \Delta T_{aL} = \frac{f\lambda \Delta T_i + k\Delta T_{aO}}{f\lambda + k} \quad (1)$$

$$\Delta T_{aO} = \frac{(f(1-f)\lambda + k)\lambda \Delta T_i + k_{as}(1-f)(f\lambda + k)\Delta T}{(f(1-f)\lambda + k)\lambda + k_{as}(1-f)(f\lambda + k)} \quad (2)$$

$$C_m \frac{d\Delta T}{dt} = \Delta Q - \lambda \Delta T - \Delta F \quad (3)$$

where  $\Delta T_L$ ,  $\Delta T_{aL}$ ,  $\Delta T_{aO}$  and  $\Delta T(t)$  are temperature changes for the land, atmosphere over land, atmosphere over ocean and

oceanic mixed layer induced by a change in thermal forcing  $\Delta Q(t)$ ,  $f$  is the fraction of the globe covered by land ( $f \approx 0.29$ ),  $\lambda$  is the climate feedback parameter<sup>10</sup>,  $k$  is a land-ocean heat transfer coefficient,  $k_{as}$  is an air-sea heat exchange coefficient, and  $\Delta T_i(t)$  is the instantaneous equilibrium temperature change

$$\Delta T_i = \frac{\Delta Q}{\lambda} \approx \Delta T_{2\times} \frac{\ln(C/C_0)}{\ln 2} \quad (4)$$

for CO<sub>2</sub> change from  $C_0$  to  $C(t)$  (ref. 11). In equation (3),  $C_m$  is the effective bulk heat capacity ( $C_m = \gamma\rho ch$ ) where

$$\gamma = \frac{(1-f)(k + \lambda f)}{k + \lambda f(1-f)} + \frac{\lambda}{k_{as}} \quad (5)$$

and  $h$  is the depth of the mixed layer in which the density and specific heat capacity are  $\rho$  and  $c$  respectively.  $\Delta F$  is the change in heat flux at the bottom of the mixed layer ( $z=0$ )

$$\Delta F = -\gamma\rho c\kappa \left( \frac{\partial \Delta T_0}{\partial z} \right)_{z=0} \quad (6)$$

where  $\kappa$  is the thermal diffusivity of the deep ocean ( $z > 0$ ) whose temperature change due to  $\Delta Q$  is  $\Delta T_0(z, t)$ .  $z$  is depth below the bottom of the mixed layer.  $\Delta T_0$  is determined by

$$\frac{\partial \Delta T_0}{\partial t} = \kappa \frac{\partial^2 \Delta T_0}{\partial z^2} \quad (7)$$

With the initial condition  $\Delta T_0(z, 0) = 0$  and the boundary conditions  $\Delta T_0(0, t) = \Delta T(t)$  and  $\Delta T_0(z, t) \rightarrow 0$  as  $z \rightarrow \infty$ , the solution to equation (7) can be approximated to give

$$\Delta F = \gamma\mu\rho ch \frac{\Delta T}{\sqrt{\tau_d t}} \quad (8)$$

where  $\tau_d = \pi h^2/\kappa$  is a characteristic time for exchange between the mixed layer and the deeper ocean;  $\tau_d$  varies between 5 and 10 yr for realistic values of  $h$  and  $\kappa$ . The constant  $\mu$  can be determined by comparing results from an analytical solution of equation (9) below, with those from a full numerical integration of the coupled equations (3), (6) and (7). The value of  $\mu$  depends to some degree on the assumed forcing ( $\Delta Q(t)$ ), but is insensitive to variations in  $\kappa$ ,  $\lambda$  and  $h$ . The validity of this approximation is discussed below (see Table 1).

Since  $\lambda \ll k_{as}$  (ref. 10),  $\Delta T_{aO} \approx \Delta T$  in accord with observations (ref. 6), and the  $\lambda/k_{as}$  term in the expression for  $\gamma$  may be ignored. Two limiting cases give the bounds on  $\gamma$ . For  $k=0$  (no land-sea heat exchange),  $\gamma=1$ , which also corresponds to an ocean-covered Earth. For  $k \rightarrow \infty$  (infinitely fast land-sea heat exchange),  $\gamma=1-f \approx 0.71$ , which corresponds to the fraction of the globe covered by ocean. For realistic values of  $\lambda$  and  $k$ ,  $\gamma$  lies between 0.72 and 0.75.

Substitution of equation (8) into equation (3) gives an ordinary differential equation describing  $\Delta T(t)$ :

$$\gamma \frac{d\Delta T}{dt} + \Delta T \left\{ \frac{1}{\tau_f} + \frac{\mu\gamma}{\sqrt{\tau_d t}} \right\} = \frac{\Delta Q}{\rho ch} \quad (9)$$

where  $\tau_f = \rho ch/\lambda$  is the response time for step-function forcing of a thermally isolated mixed layer with no land-sea heat exchange, that is,  $\Delta F=0$  and  $\gamma=1$ .  $\tau_f$  ranges between 4 and 13 yr for realistic values of  $h$  and  $\lambda$ .

Equation (9) can be solved exactly for a number of different forms of the forcing function  $\Delta Q$ . We will consider  $\Delta Q = a$ , giving the transient response to a step-function CO<sub>2</sub> change (the 'switch-on' experiment<sup>12</sup>), and  $\Delta Q = bt \exp(\alpha t)$ ;  $a$ ,  $b$  and  $\alpha$  are arbitrary constants. Since the radiative response is approximately proportional to the logarithm of the concentration (ref. 11 and equation (4) above),  $\alpha \neq 0$  corresponds to an exponentially increasing CO<sub>2</sub> concentration while  $\alpha \approx 0$ , for appropriate  $b$  and  $\alpha$ , corresponds closely to observed changes of CO<sub>2</sub> over the past 130 yr.



For the switch-on experiment,  $\Delta T$  tends to  $\Delta T_e$  for large  $t$  where

$$\Delta T_e = a/\lambda \quad (10)$$

The solution to equation (9) is then

$$\frac{\Delta T}{\Delta T_e} = 1 - e^{-W} - 2G \quad (11)$$

where  $G = G(Z, N) = N[D(Z+N) - e^{-W}D(N)]$ ,  $W = W(Z, N) = Z^2 + 2NZ$ ,  $Z^2 = t/(\gamma\tau_f)$  and  $N^2 = \gamma\mu^2\tau_f/\tau_d$ . In  $G(Z, N)$ ,  $D$  is Dawson's integral<sup>13</sup>,  $D(z) = e^{-z^2} \int_0^z e^{u^2} du$ . The parameter  $N$  depends on  $\mu$  which takes the value 1.4 for step-function forcing.

The switch-on case is important because it has been used by a number of investigators to study the transient response characteristics of the climate system. The speed of response, and the influence of oceanic thermal inertia, can be characterized by the e-folding time,  $\tau_e$ , defined by  $\Delta T(\tau_e) = (1 - e^{-1})\Delta T_e$ . If  $Z_e^2 = \tau_e/(\gamma\tau_f)$ , then  $\tau_e$  is determined by

$$e^{-W_e} + 2G(Z_e, N) = e^{-1} \quad (12)$$

For large  $N$  the solution is  $Z_e \approx (e-1)N$  (using  $D(u) \sim 1/2u$ ), an excellent approximation (error in  $Z_e$  less than 5%) for  $N > 1.5$ , that is, for almost all values of  $N$  likely to be encountered. Hence

$$\tau_e \approx \gamma^2\mu^2(e-1)^2(\tau_f)^2/\tau_d \approx 47.8\gamma^2\mu^2\kappa/\lambda^2 \quad (13)$$

for  $\kappa$  in  $\text{cm}^2\text{s}^{-1}$  and  $\lambda$  in  $\text{Wm}^{-2}\text{K}^{-1}$ . If we consider the case of a step-function doubling of  $\text{CO}_2$  concentration, then (from equation (10))  $\lambda = \Delta Q_{2\times}/\Delta T_{2\times}$  where  $\Delta Q_{2\times} \approx 4.3\text{ Wm}^{-2}$  (ref. 3). Since  $\mu = 1.4$ , equation (13) becomes

$$\tau_e \approx 5.08\gamma^2\kappa(\Delta T_{2\times})^2 \quad (14)$$

$\tau_e$  is independent of the mixed layer depth,  $h$ . This analytical result can be compared with the numerical calculations of Hansen *et al.*<sup>3</sup>. For  $\kappa = 1\text{ cm}^2\text{s}^{-1}$  and  $\Delta T_{2\times} = 4.2, 3.0, 2.0^\circ\text{C}$ , these authors obtain  $\tau_e = 102, 55, 27$  yr using  $\gamma = 1$ . Equation (14) gives  $\tau_e = 90, 46, 20$  yr, in close agreement. For  $\gamma = 0.75$  (see above),  $\tau_e$  ranges between 6 and 174 yr for  $\kappa = 1-3\text{ cm}^2\text{s}^{-1}$  and  $\Delta T_{2\times} = 1.5-4.5^\circ\text{C}$ . There is thus a considerable range of uncertainty in  $\tau_e$  resulting from uncertainties in the magnitude of the  $\text{CO}_2$ -doubling temperature change and in sub-mixed-layer mixing.

The e-folding time demonstrates that oceanic thermal inertia has a significant effect in determining the response of the climate system to external forcing. However, the results of a switch-on experiment are transferable to the case of steadily increasing  $\text{CO}_2$  concentration only through a convolution integral<sup>4</sup>. We therefore consider the 'continuous increase' experiment with time-dependent forcing.

Forcing of the form  $\Delta Q = bt \exp(\alpha t)$  corresponds to  $\text{CO}_2$  variations given by

$$C(t) = C_0 \exp(Bt e^{\alpha t}) \quad (15)$$

where  $C_0$  is the initial, pre-industrial concentration and  $B = b \ln 2/\Delta Q_{2\times}$ . Observed  $\text{CO}_2$  changes can be fitted to this form. For example, if  $C_0 = 270$  parts per million by volume (p.p.m.v.) in 1850<sup>14</sup>, and  $C = 315$  p.p.m.v. in 1958 and 338 p.p.m.v. in 1980<sup>15</sup>, then  $B = 5.59 \times 10^{-4}$  and  $\alpha = 8.69 \times 10^{-3}\text{ yr}^{-1}$ .

The solution to equation (9) for  $\Delta Q = bt \exp(\alpha t)$  is

$$\frac{\Delta T}{\Delta T_i} = \frac{\gamma\tau_f}{\beta^2 t} [\tilde{Z}^2 - \tilde{N}\tilde{Z} - (1 - \tilde{N}^2)(1 - e^{-\tilde{W}}) + (3 - 2\tilde{N}^2)\tilde{G}] \quad (16)$$

where  $\Delta T_i$  is the instantaneous equilibrium temperature change at time  $t$  (equation (4)),  $\beta = 1 + \alpha\gamma\tau_f$ ,  $\tilde{Z}^2 = \beta Z^2$ ,  $\tilde{N}^2 = N^2/\beta$ ,  $\tilde{W} = W(\tilde{Z}, \tilde{N})$  and  $\tilde{G} = G(\tilde{Z}, \tilde{N})$ . The case  $\beta = 1$  (that is,  $\alpha = 0$ ,  $\tilde{W} = W$ ,  $\tilde{G} = G$ ) corresponds to linear thermal forcing due to an exponentially increasing  $\text{CO}_2$  concentration. The departure of  $\Delta T/\Delta T_i$  from unity is a measure of the degree of disequilibrium due to oceanic thermal inertia effects.

Since the initial  $\text{CO}_2$  level and the history of past  $\text{CO}_2$  variations appear in equation (16) only through the  $\alpha$  term in  $\beta$ ,

and since  $\beta$  is always close to 1, the degree of disequilibrium must be insensitive to the details of past  $\text{CO}_2$  changes. This is demonstrated in Table 1. Insensitivity to the initial  $\text{CO}_2$  level can be seen by comparing  $\Delta T/\Delta T_i$  values for various values of  $C_0$ , and insensitivity to the  $\text{CO}_2$  history for given  $C_0$  can be seen by the proximity of  $\Delta T/\Delta T_{\text{lin}}$  (where  $\Delta T_{\text{lin}}$  corresponds to  $\Delta T$  for  $\alpha = 0$ ) to unity. The degree of disequilibrium, however, does vary markedly with  $\kappa$  and  $\Delta T_{2\times}$ .

Equation (16) implies that the response  $\Delta T(t)$  lags behind the instantaneous equilibrium response by a time-varying amount  $L$  which can be defined by

$$\Delta T(t) = \Delta T_i(t - L) \quad (17)$$

It can easily be shown that

$$\frac{\Delta T(t)}{\Delta T_i(t)} = \left(1 - \frac{L}{t}\right) e^{-\alpha L} \quad (18)$$

so that, for  $\alpha \geq 0$ ,  $L$  is a maximum in the linear forcing case. For this case ( $\alpha = 0$ )  $L (= L_{\text{lin}})$  can be related to the switch-on e-folding time  $\tau_e$  (equation (14)) by substituting equation (16) into (18) and using  $D(u) \sim 1/2u$ :

$$L_{\text{lin}} \approx 0.91\sqrt{\tau_e} - 0.84\tau_e(1 - 0.91\xi) + \gamma\tau_f(1 - 1.37\xi) \quad (19)$$

where  $\xi = (0.91 + \sqrt{t/\tau_e})^{-1}$ .  $L_{\text{lin}}$  is therefore independent of the initial  $\text{CO}_2$  level and the timescale for  $\text{CO}_2$  increase (see Table 1).

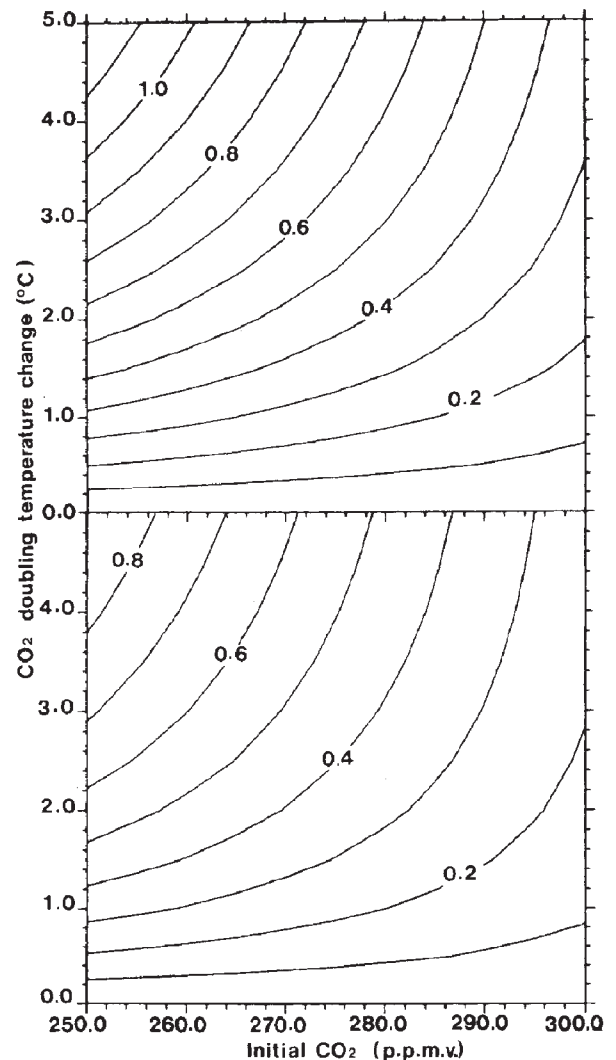


Fig. 1 Isolines of temperature change to 1980 ( $\text{CO}_2$  level of 338 p.p.m.v.) as a function of the  $\text{CO}_2$ -doubling temperature change and the 1850 initial  $\text{CO}_2$  level for two extreme pairs of diffusivity and mixed layer depth. Lower diagram,  $\kappa = 1\text{ cm}^2\text{s}^{-1}$ ,  $h = 70\text{ m}$ ; upper diagram,  $\kappa = 3\text{ cm}^2\text{s}^{-1}$ ,  $h = 110\text{ m}$ . Results are based on a full numerical solution of equations (3), (6) and (7).

For  $\alpha \neq 0$ , however, the lag is strongly dependent on  $\alpha$  and  $C_0$ , the parameters characterizing the history of past  $\text{CO}_2$  variations. The sensitivity to  $\alpha$  can be seen by comparing  $L$  with  $L_{\text{lin}}$  in Table 1, while the sensitivity to  $C_0$  can be seen directly in this table. Because of this, and because  $C_0$  is uncertain<sup>14</sup>, the lag time is not a particularly good parameter to use as an indicator of oceanic thermal inertia effects. Table 1 also shows that  $L$  is strongly dependent on  $\kappa$  and  $\Delta T_{2x}$ , the values of which are also uncertain.

The most important result shown in Table 1 is the wide range of uncertainty in the disequilibrium ratio,  $\Delta T/\Delta T_i$ , 0.42 to 0.79 for  $C_0 = 270$  p.p.m.v.  $\Delta T/\Delta T_i$  decreases as  $\Delta T_{2x}$  increases, in accord with the results of Siegenthaler and Oeschger<sup>9</sup> (who used  $\Delta T_{2x} = 2.0^\circ\text{C}$  and  $3.2^\circ\text{C}$  with  $\kappa = 1\text{ cm}^2\text{ s}^{-1}$ ), and as  $\kappa$  increases, in accord with Hansen *et al.*<sup>3</sup> (who used  $\kappa = 1$  and  $2\text{ cm}^2\text{ s}^{-1}$  with  $\Delta T_{2x} = 4.2^\circ\text{C}$ ). Hansen *et al.*'s values of  $\Delta T/\Delta T_i$  are noticeably less than ours because they used  $\gamma = 1$ . If the climate sensitivity is as high as recent model results suggest (refs 2-4) then  $\Delta T/\Delta T_i \approx 0.5$  and a substantial future warming must occur even in the absence of further  $\text{CO}_2$  increases, a point already noted by Hansen *et al.*<sup>3</sup> and by Siegenthaler and Oeschger<sup>9</sup>.

The overall uncertainty is further illustrated in Fig. 1 which shows  $\Delta T(1980)$  as a function of initial  $\text{CO}_2$  level and  $\text{CO}_2$ -doubling temperature change. The warming depends strongly on the initial  $\text{CO}_2$  level.  $\Delta T$  values in Fig. 1 are compatible with the observed global mean warming of around  $0.5^\circ\text{C}$  over the past 100 yr, especially when one considers the uncertainties in the observational record<sup>5,6</sup>. For  $260 < C_0 < 280$  p.p.m.v. (ref. 14) and  $1.5 < \Delta T_{2x} < 4.5^\circ\text{C}$  (ref. 1),  $\Delta T$  ranges between 0.26 and  $0.96^\circ\text{C}$ . If observed temperature changes in the range  $0.4$ – $0.6^\circ\text{C}$  were to be explained solely in terms of  $\text{CO}_2$  changes then, for  $260 < C_0 < 280$  p.p.m.v., the implied  $\Delta T_{2x}$  range is  $1.3$ – $11.5^\circ\text{C}$ . A consideration of transient response effects, therefore, helps to remove the apparent conflict noted in ref. 16 between recent model results<sup>2-4</sup> and observations<sup>5,6</sup> (a point noted by Hansen *et al.*<sup>3</sup>). The high upper limit to the implied  $\Delta T_{2x}$  value (that is,  $11.5^\circ\text{C}$ , corresponding to  $\Delta T = 0.6^\circ\text{C}$ ,  $C_0 = 280$  p.p.m.v.,  $\kappa = 3\text{ cm}^2\text{ s}^{-1}$ ,  $h = 110\text{ m}$ ) requires comment.  $\Delta T_{2x}$  affects  $\Delta T(t)$  in two competing ways; through  $\Delta T_i(t)$ , which increases with increasing  $\Delta T_{2x}$ , and through the disequilibrium ratio,  $\Delta T/\Delta T_i$ , which decreases as  $\Delta T_{2x}$  increases. Therefore, as Fig. 1 shows, the 1980 value of  $\partial\Delta T/\partial\Delta T_{2x}$  becomes small for large  $\Delta T_{2x}$  (particularly for large  $\kappa$ ), so that  $\Delta T(1980)$  can be quite insensitive to changes in  $\Delta T_{2x}$ . The  $11.5^\circ\text{C}$  upper limit for  $\Delta T_{2x}$  is in this insensitive region, but its value is sensitive to  $C_0$  and  $\kappa$ , and

to the chosen history of  $\text{CO}_2$  variations. Thus, it is the qualitative conclusion that a large value of  $\Delta T_{2x}$  could be consistent with observed  $\Delta T$  values which is important, rather than the specific numerical value obtained here.

An additional source of uncertainty becomes evident retrospectively from the analytical solution. Because of the lag in the response due to the thermal inertia of the oceans, we can be fairly sure that the climate system was not initially in equilibrium as we have assumed in our calculations. The subsequent warming does depend on whether the initial global-mean temperature was above or below the equilibrium temperature, that is on the thermal forcing history before 1850. In the absence of information about the degree or even direction of the initial disequilibrium, we must neglect this confounding issue.

We have considered only  $\text{CO}_2$  in defining the thermal forcing function  $\Delta Q(t)$ . Recent increases in the concentrations of other trace gases (such as methane and chlorofluorocarbons) may have added noticeably to the forcing<sup>3,17</sup>, contributing perhaps as much as  $0.2^\circ\text{C}$  to the overall warming since 1960<sup>18</sup>. The  $\text{CO}_2$  contribution to the observed global mean warming of  $0.4$ – $0.6^\circ\text{C}$  over the past 100 yr would therefore be only  $0.2$ – $0.4^\circ\text{C}$ . For  $260 < C_0 < 280$  p.p.m.v., this implies a  $\Delta T_{2x}$  range of only  $0.6$ – $3.1^\circ\text{C}$  (see Fig. 1). Recent ice-core evidence, however, suggests that the  $260$ – $280$  p.p.m.v. initial  $\text{CO}_2$  range may be too low. Neftali *et al.*<sup>19</sup> give new data with concentrations around 290 p.p.m.v. in the late nineteenth century. Initial levels of 270 and 290 p.p.m.v. give  $\Delta T(1980)$  values which differ by about  $0.2^\circ\text{C}$ , largely compensating for the trace gas effect. This higher initial  $\text{CO}_2$  level may, therefore, be necessary in order to ensure compatibility between model results and observations.

These results cannot be construed as proof of the detection of  $\text{CO}_2$  effects on climate, although they do help to bring model results more in line with observations. Detection requires a statistically significant warming above the noise level of natural interannual and interdecadal temperature fluctuations<sup>20,21</sup>, together with a convincing attribution of at least part of such warming to carbon dioxide. These statistical aspects, together with problems of data reliability and representativeness, and our present inadequate understanding of the processes other than  $\text{CO}_2$  that can cause global climate change, make it impossible to attribute the observed warming unequivocally to  $\text{CO}_2$ . In fact, transient response effects make detection even more difficult because, by lowering the signal expected at any given time, the signal-to-noise ratio is correspondingly lowered. The insensitivity of  $\Delta T(1980)$  to  $\Delta T_{2x}$  for large  $\Delta T_{2x}$  means that it may be very difficult to determine  $\Delta T_{2x}$  from observational data.

Table 1 Disequilibrium ratios and lags (yr)

| $\Delta T_{2x}$ | $C_0$                 | $\kappa$                         | 250        |            |            | 270        |            |            | 290        |            |            |
|-----------------|-----------------------|----------------------------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|
|                 |                       |                                  | 1          | 2          | 3          | 1          | 2          | 3          | 1          | 2          | 3          |
| 1.5             | $\Delta T/\Delta T_i$ | $\Delta T/\Delta T_i$            | 0.81(0.79) | 0.76(0.73) | 0.72(0.69) | 0.79(0.79) | 0.73(0.73) | 0.69(0.69) | 0.74(0.77) | 0.68(0.72) | 0.63(0.68) |
|                 |                       | $\Delta T/\Delta T_{\text{lin}}$ | 0.97(0.96) | 0.97(0.95) | 0.96(0.94) | 0.94(0.95) | 0.93(0.94) | 0.92(0.94) | 0.88(0.93) | 0.87(0.92) | 0.85(0.92) |
|                 |                       | $L$                              | 18(19)     | 23(25)     | 27(29)     | 14(14)     | 19(19)     | 22(22)     | 11(10)     | 14(12)     | 16(14)     |
|                 |                       | $L_{\text{lin}}$                 | 22(23)     | 28(29)     | 33(34)     | 22(23)     | 28(29)     | 33(34)     | 22(23)     | 28(29)     | 33(34)     |
| 3.0             | $\Delta T/\Delta T_i$ | $\Delta T/\Delta T_i$            | 0.68(0.65) | 0.60(0.58) | 0.56(0.53) | 0.64(0.64) | 0.57(0.57) | 0.52(0.53) | 0.58(0.63) | 0.51(0.56) | 0.46(0.51) |
|                 |                       | $\Delta T/\Delta T_{\text{lin}}$ | 0.96(0.93) | 0.95(0.92) | 0.95(0.91) | 0.91(0.92) | 0.90(0.91) | 0.89(0.90) | 0.82(0.89) | 0.80(0.88) | 0.79(0.88) |
|                 |                       | $L$                              | 31(34)     | 39(42)     | 45(47)     | 26(25)     | 32(32)     | 37(36)     | 19(17)     | 24(21)     | 27(24)     |
|                 |                       | $L_{\text{lin}}$                 | 38(39)     | 48(48)     | 54(54)     | 38(39)     | 48(48)     | 54(54)     | 38(39)     | 48(48)     | 54(54)     |
| 4.5             | $\Delta T/\Delta T_i$ | $\Delta T/\Delta T_i$            | 0.58(0.56) | 0.50(0.48) | 0.45(0.43) | 0.54(0.55) | 0.47(0.47) | 0.42(0.43) | 0.48(0.53) | 0.41(0.46) | 0.36(0.41) |
|                 |                       | $\Delta T/\Delta T_{\text{lin}}$ | 0.95(0.91) | 0.94(0.90) | 0.94(0.89) | 0.89(0.90) | 0.88(0.89) | 0.87(0.88) | 0.79(0.86) | 0.77(0.86) | 0.76(0.85) |
|                 |                       | $L$                              | 42(45)     | 52(54)     | 57(60)     | 35(34)     | 43(42)     | 48(47)     | 26(23)     | 32(28)     | 35(31)     |
|                 |                       | $L_{\text{lin}}$                 | 51(51)     | 61(61)     | 67(67)     | 51(51)     | 61(61)     | 67(67)     | 51(51)     | 61(61)     | 67(67)     |

Values represent variations in the disequilibrium ratio ( $\Delta T/\Delta T_i$ ) and the lag ( $L$ ) in 1980 for  $\gamma = 0.71$  and various values of the diffusivity ( $\kappa = 1$ – $3\text{ cm}^2\text{ s}^{-1}$ , see refs 4, 9, 22, 23), the  $\text{CO}_2$ -doubling temperature change ( $\Delta T_{2x} = 1.5$ – $4.5^\circ\text{C}$ , see ref. 1) and the initial (1850)  $\text{CO}_2$  level ( $C_0 = 250$ – $290$  p.p.m.v., a range which spans the most likely range of  $260$ – $280$  p.p.m.v. given in ref. 14).  $\Delta T$  and  $L$  correspond to  $\text{CO}_2$  variations given by equation (15) fitted through 315 p.p.m.v. in 1958 and 338 p.p.m.v. in 1980<sup>15</sup>, while  $\Delta T_{\text{lin}}$  and  $L_{\text{lin}}$  correspond to  $\text{CO}_2$  variations given by equation (15) with  $\alpha = 0$  fitted through 338 p.p.m.v. in 1980 (that is, to linear radiative forcing).  $\Delta T$  and  $L$  are relatively insensitive to variations in  $h$ ;  $h = 70\text{ m}$  has been used here (ref. 3). The values shown are based on a full numerical solution of equations (3), (6) and (7), while the bracketed figures are those based on the approximate analytical solution (equation (16)) with  $\mu = 2.2$  in the linear case and  $\mu = 2.7$  otherwise. In the nonlinear case the optimum value of  $\mu$  depends on  $C_0$ , but we have used the value appropriate to  $C_0 = 270$  p.p.m.v. throughout.



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## Methane flux from northern peatlands

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The concentration of methane (CH<sub>4</sub>) in the global troposphere is increasing. Ambient air measurements document an approximate rate of increase of 1-2% yr<sup>-1</sup> over the past decade<sup>1-4</sup>. Measurements of CH<sub>4</sub> in air bubbles trapped in polar ice indicate that tropospheric concentrations of CH<sub>4</sub> several hundred years ago may have been ~45% of present levels<sup>5-7</sup>. To understand and assess possible causes of the atmospheric CH<sub>4</sub> increase requires improved quantitative knowledge of global sources and sinks of CH<sub>4</sub>. Previous attempts to estimate sources of atmospheric CH<sub>4</sub>, based on very few measurements, have suggested that natural and agricultural wetlands are major sources<sup>8,9</sup>. The major wetland regions of the world are in boreal, low Arctic and tropical ecosystems<sup>10</sup>. It is these regions, particularly in peatland habitats where major accumulations of organic materials occur under anaerobic conditions, that should be significant sources of global tropospheric CH<sub>4</sub>. The most extensive peatlands in the world occur in the boreal taiga zone between ~45° and 65° N latitude. More than 95% of world peat resources occur in the Soviet Union, Canada, the United States, Sweden, Norway, Finland and the United Kingdom<sup>10</sup>. We report here the first survey of CH<sub>4</sub> flux from northern peatlands of the United States. Emission rates ranged from 0.003 to 1.94 g CH<sub>4</sub> m<sup>-2</sup> day<sup>-1</sup>, with half of these values between 0.1 and 0.4 g CH<sub>4</sub> m<sup>-2</sup> day<sup>-1</sup>. The frequency distribution is log normal (Fig. 1) and the mean emission rate is 0.337 g CH<sub>4</sub> m<sup>-2</sup> day<sup>-1</sup>. Such fluxes are higher than most values reported for other ecosystems, suggesting that northern peatlands may be an important source of global tropospheric CH<sub>4</sub>.

In an effort to improve our understanding of wetlands as a source of atmospheric CH<sub>4</sub>, which should aid in the interpretation of global distributions and trends, we developed a portable system for rapid, direct measurement of CH<sub>4</sub> flux in remote wetland environments. The system consists of a gas-filter-correlation (GFC) infrared detector<sup>11</sup> coupled to a controlled environment chamber. Flux measurements were made in a closed-chamber mode with air continuously recirculated from the sampling chamber over the surface peat and vegetation, then into the GFC and back to the chamber; CH<sub>4</sub> concentration was measured continuously. Continuous measurement of CH<sub>4</sub> in the chamber enables us to separate 'pulses' related to any human disturbance of the bog from the natural efflux. For a typical 15-min continuous sampling and analysis period, this system has a flux detection limit of 1 × 10<sup>-4</sup> g CH<sub>4</sub> m<sup>-2</sup> day<sup>-1</sup>. Calibration was carried out in the field using certified CH<sub>4</sub> standards. Details of instrument design, calibration, and field testing are given elsewhere<sup>11,12</sup>.

Our survey of CH<sub>4</sub> emissions from northern wetlands was conducted in August 1983, during daylight hours. Four sites were in the Marcell Experimental Forest, north-central Minnesota (47°32' N, 93°28' W), where a single watershed often consists of a central peatland surrounded by forested uplands. Characteristic plants of the Marcell fens and bogs are given by Boelter and Verry<sup>13</sup>. Upland soils over slightly calcareous glacial debris constitute 74% of the Marcell Forest surface, peatlands comprise 21%, and open water the remaining 5% (ref. 14). Peat deposits consist primarily of *Sphagnum* peat at the surface with woody, sedge, and aquatic plant residues at depth<sup>15</sup>. Three of our Marcell sites are perched ombrotrophic bogs; their water is derived almost entirely from precipitation (that is, WS-1, WS-2, and WS-4 in Table 1). Site WS-3 is a minerotrophic shrub/sedge fen; its water is derived primarily from groundwater. The hydrology and chemistry of these bogs have been studied in detail elsewhere<sup>13</sup>. The ombrotrophic bogs have low concentrations of dissolved ions, with a conductance 51 ± 13 μS cm<sup>-1</sup> at 25 °C and an average pH of 3.6 ± 0.3. The minerotrophic fen (WS-3) has high concentrations of dissolved ions from reactions of groundwater with soil, conductance 125 ± 48 μS cm<sup>-1</sup>, pH 6.5 ± 0.3 (ref. 14).

We also studied CH<sub>4</sub> flux from 21 separate sites in an area where a weakly minerotrophic fen surrounds a small ombrotrophic bog, 10.5 km west of Zerkel, Minnesota (47°10' N, 95°24' W). The vegetation includes a central pond with *Brasenia schreberi*, surrounded by a floating mat of *Carex lasiocarpa* and *Vaccinium macrocarpon*. The sedge mat is being invaded on its landward side by bog mosses such as *Sphagnum angustifolium*, *S. subsecundum* and *S. magellanicum* and by the vascular plants *Vaccinium oxycoccus*, *Potentilla palustris*, *Dulichium arundinaceum* and *Scheuchzeria palustris*. Closer to the upland is a zone of low leatherleaf shrubs (*Chamaedaphne calyculata*) and several bog mosses, including *Sphagnum rubellum*, *S. magellanicum*, *S. papillosum*, *S. angustifolium* and *S. fuscum*, together with *Vaccinium oxycoccus*, *Scheuchzeria palustris*, and *Andromeda glaucophylla*. We also sampled ombrotrophic sites at this location inside and outside a small stand of black spruce (*Picea mariana*) associated with *Chamaedaphne calyculata*, *Ledum groenlandicum*, *Carex trisperma*, *Eriophorum spissum* and

Table 1 Methane flux from peatland sites in northern Minnesota

| Site                         | No. | CH <sub>4</sub> flux (g CH <sub>4</sub> m <sup>-2</sup> day <sup>-1</sup> ) |             |
|------------------------------|-----|-----------------------------------------------------------------------------|-------------|
|                              |     | Average                                                                     | Range       |
| Marcell Experimental Forest  |     |                                                                             |             |
| WS-1 (bog)                   | 3   | 0.156                                                                       | 0.127-0.206 |
| WS-2 (bog)                   | 9   | 0.047                                                                       | 0.019-0.174 |
| WS-3 (fen)                   | 3   | 0.004                                                                       | 0.003-0.005 |
| WS-4 (bog)                   | 6   | 0.194                                                                       | 0.033-0.468 |
| Zerkel fen and bog           | 18  | 0.419                                                                       | 0.060-1.943 |
| Wild Rice River sedge meadow | 1   | 0.664                                                                       | —           |
| Lake Itasca                  |     |                                                                             |             |
| Wild rice bed                | 4   | 0.493                                                                       | 0.127-0.883 |
| Shoreline fen                | 2   | 0.165                                                                       | 0.158-0.171 |

the mosses *Sphagnum angustifolium*, *S. magellanicum*, *S. rubellum*, *S. fuscum*, *Aulacomnium palustre*, *Calliergon stramineum* and *Ptilium crista-castrensis*. This location is more fully described by Joyal<sup>16</sup>. The Zerkel sites ranged in pH from circumneutral in the pond and the outer part of the floating sedge mat (pH 6.1–7.3) to distinctly acid in the ombrotrophic area of black spruce and *Sphagnum* (pH 4.1–4.4). Conductivities ranged from 29 to 98  $\mu\text{S cm}^{-1}$  in various parts of the pond and floating mat, and from 43 to 108  $\mu\text{S cm}^{-1}$  in the zone of leatherleaf shrubs. In the spruce stand, we have one measurement of 60  $\mu\text{S cm}^{-1}$ .

For comparison with other wetland habitats of the region, we measured the  $\text{CH}_4$  flux in the Wild Rice River sedge meadow, located ~5 km west of Zerkel, and dominated by *Carex lacustris* and *Calamagrostis canadensis*, and in a stand of wild rice (*Zizania aquatica*) and shoreline fen adjacent to Lake Itasca, Minnesota (47°15' N, 95°10' W), where the pH ranged from 6.9 to 7.5. The combined set of sites studied are representative of major types of wetlands found in boreal North American ecosystems.

Our results are summarized in Table 1. Note that average  $\text{CH}_4$  efflux rates are much the same across a wide range of peatland sites, from circumneutral lake edges and marginal sedge fens through increasingly acid sedge/*Sphagnum* and leatherleaf/*Sphagnum* communities to an ombrotrophic bog community of black spruce and *Sphagnum*. This is particularly interesting because laboratory studies using Minnesota peats indicated that pH=6 is optimal for  $\text{CH}_4$  production<sup>17</sup>. This difference between laboratory and field observations may well be explained by all variables except pH being held constant in the laboratory. At two locations with significantly lower  $\text{CH}_4$  flux (Marcell WS-2 and WS-3) our qualitative field observations suggest that differences in peatland hydrology may be important in controlling  $\text{CH}_4$  production. The Marcell fen WS-3, which had by far the lowest average daily  $\text{CH}_4$  flux, 0.004  $\text{g m}^{-2} \text{ day}^{-1}$ , was the only site with visible water flow in surface peat derived from active groundwater input. Thus, we hypothesize that relatively stagnant conditions were present at the other sites studied (conditions at WS-2 were intermediate) which favoured higher rates of methanogenesis. Methane fluxes from the sedge meadow, wild rice, and shoreline fen were similar to rates measured in the bogs.

The distribution of all  $\text{CH}_4$  flux measurements made in Minnesota peatlands (Fig. 1) indicates that emission rates ranged over three orders of magnitude. Approximately half of our  $\text{CH}_4$  measurements fell within the limited range of 0.1–0.4  $\text{g CH}_4 \text{ m}^{-2} \text{ day}^{-1}$ . The logarithmic nature of the frequency distribution of flux rates is of considerable interest. We speculate that the lowest values represent a combination of low decay rates and largely molecular diffusion from the bog surface, whereas the high values, above ~0.4  $\text{g CH}_4 \text{ m}^{-2} \text{ day}^{-1}$ , represent scattered 'hotspots' of decaying, recently dead roots or small animals and the possible buildup of methane bubbles.

The only comparable data of which we are aware are from an ombrotrophic *Sphagnum* bog in subarctic northern Sweden<sup>18</sup> where  $\text{CH}_4$  emissions ranged from 0.0003 to 0.058  $\text{g CH}_4 \text{ m}^{-2} \text{ day}^{-1}$ , a minerotrophic peatland in Sweden<sup>18</sup> that emitted 0.054–0.95  $\text{g CH}_4 \text{ m}^{-2} \text{ day}^{-1}$ , and an acid *Sphagnum* bog in the Pennine mountains of England<sup>19</sup> with a range of 0.004–0.03  $\text{g CH}_4 \text{ m}^{-2} \text{ day}^{-1}$ . The generally higher emission rates from Minnesota peatlands are probably due, in part at least, to higher soil temperatures that ranged from 14 to 20 °C at 6 cm, as compared with 8 to 16 °C in Sweden and ~11 °C in the British bog<sup>20</sup> during late summer.

Comparison of our flux data with data on other biogenic  $\text{CH}_4$  sources indicates that northern wetlands should be considered a potentially significant source of global tropospheric  $\text{CH}_4$ . For example, our Minnesota flux data are similar to the average emissions of 0.25  $\text{g CH}_4 \text{ m}^{-2} \text{ day}^{-1}$  from California rice paddies<sup>21</sup> and 0.1  $\text{g CH}_4 \text{ m}^{-2} \text{ day}^{-1}$  from rice paddies in Spain<sup>22</sup>, and higher than emissions of 0.01–0.09  $\text{g CH}_4 \text{ m}^{-2} \text{ day}^{-1}$  from undisturbed freshwater cypress swamps<sup>23</sup>. Two additional factors that determine the global significance of biogenic sources

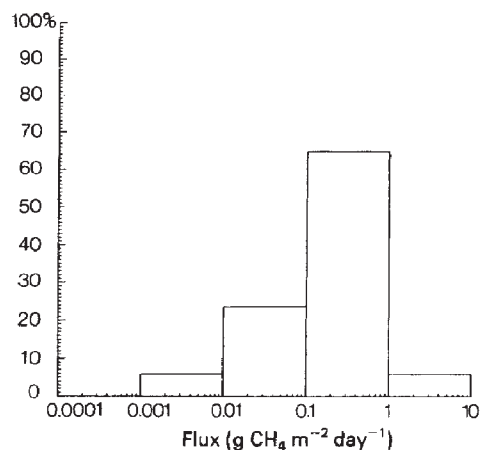


Fig. 1 Histogram of  $\text{CH}_4$  flux data from Minnesota peatlands. There were 51 observations which gave a mean flux of  $0.337 \pm 0.076 \text{ g CH}_4 \text{ m}^{-2} \text{ day}^{-1}$  and a median of 0.173.

of  $\text{CH}_4$  are the geographical extent of the source and the duration of emissions. The surface area of boreal and Arctic peatlands and wetlands is not well known. Recently published estimates of these source areas<sup>24</sup> are: boreal bogs and peatland =  $1.2 \times 10^6 \text{ km}^2$ , tundra wet sedge =  $1.0 \times 10^6 \text{ km}^2$ , and tundra tussock =  $0.9 \times 10^6 \text{ km}^2$ . The area of other potentially significant source regions such as wet rice agriculture and Amazonian floodplain are  $\sim 1.1 \times 10^6 \text{ km}^2$  and  $0.06 \times 10^6 \text{ km}^2$ , respectively. All of these areas produce  $\text{CH}_4$  for 3–6 months a year, depending on climatic and cultivation cycles. We hypothesize that the combination of relatively high emission rates and large areal extent should rank northern peatlands high as a natural source for global atmospheric  $\text{CH}_4$ . Flux measurements from the great peatlands of Canada and the Soviet Union are needed to test our hypothesis.

Habitats suitable for peat development are largely a result of the most recent glacial period. Extensive peat accumulation began in Minnesota mostly between 3,000 and 4,000 years ago (ref. 25 and E.G., unpublished data). Major peatlands across Canada from British Columbia to the Atlantic Provinces exhibit, as expected, much less synchronicity in 35 basal dates compiled from a variety of sources (E.G., unpublished data). These dates range from 1,600 to 11,200 yr BP, and average 6,500 yr BP. Could the development of boreal peatlands, and their spread by swamping of large areas of surrounding uplands, contribute sufficient additional  $\text{CH}_4$  flux to result in a long-term increase in global atmospheric  $\text{CH}_4$ ?

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## A lattice statistics model for the age distribution of air bubbles in polar ice

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Measurements of CO<sub>2</sub> in bubbles in polar ice have been used to establish a pre-industrial concentration<sup>1-4</sup>. Similar measurements have been made for other atmospheric constituents<sup>5,6</sup>. However, in order to use ice-core measurements to determine the increase in CO<sub>2</sub> over the last 200 years, it is necessary to consider the time delay between the deposition of the original snow and the bubble trapping and also the distribution of trapping times over several decades<sup>7</sup>. The percolation model from lattice statistics describes the static geometrical aspects of trapping and reproduces various aspects of recent observations. The observations of large seasonal fluctuations in trapped bubble volume reflect the enhanced susceptibility to perturbations near the percolation transition. The critical exponent of the percolation probability largely determines the stability of the deconvolution of observed concentrations, indicating that the bubble deconvolution problem is less poorly posed than typical geochemical source deduction problems.

In order to represent the relation between atmospheric concentrations and bubble concentrations, a negative time variable  $z$  (in years) is introduced. The variable  $z$  increases with depth and has its origin at the present (that is, the time at which the ice core was extracted). The atmospheric concentration of CO<sub>2</sub> (or other tracer of interest)  $z$  years ago is denoted  $c(z)$ . The mean concentration in bubbles in a layer deposited  $z$  years ago is denoted  $q(z)$ . The trapping function  $R(z)$  gives the amount of air in a layer that was trapped  $z$  years after the layer was deposited and is expressed as a proportion of the total amount of gas that will be trapped. Thus  $R(z)$  is normalized to

$$\int_0^\infty R(z) dz = 1 \quad (1)$$

From the definition of  $R(z)$ , it follows that the relation between  $q$  and  $c$  is

$$q(z) = \int_0^z R(z-z')c(z') dz' \quad (2)$$

The static geometric aspects of bubble-trapping can be naturally modelled by using the percolation model of lattice statistics<sup>8-10</sup>. This model is usually studied for regular lattices and considers connected clusters in a lattice where elementary steps in possible connecting pathways are broken in a random uncorrelated manner. A key quantity in the theory is the percolation probability,  $P(p)$ , which gives the probability that a given lattice element is part of an infinite cluster of connected elements with  $p$  being the probability that an individual step in the lattice is broken. The main results of percolation theory are that there is a critical probability  $p_c$  above which  $P(p)$  is zero and that  $P(p)$  behaves as  $(p_c - p)^\beta$  for  $p \rightarrow p_c^-$ . It was pointed out by Kasteleyn and Fortuin<sup>11</sup> that the percolation model is a limiting case of the Potts models<sup>12</sup>, a family of models used in statistical mechanics to describe a range of continuous phase transitions. The property that distinguishes such transitions and makes them

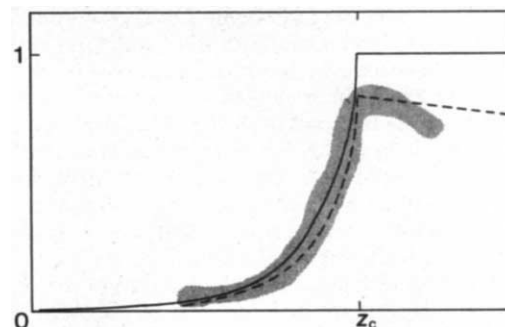


Fig. 1 A schematic representation of the cumulative trapping function as predicted by percolation theory (solid curve). The age distribution function is the derivative of this curve. The dashed curve shows the effect of a correction applied to convert the fraction trapped into bubble volume. Shading shows the range of observed volumes<sup>7</sup>, with the horizontal and vertical scales chosen to give agreement at the 50% and 100% points.

difficult to analyse is that the transition behaviour is influenced by fluctuations on all possible length scales.

Having established the connection with statistical mechanics, the techniques of the renormalization group<sup>13</sup> in statistical mechanics can be used to justify earlier conjectures of universality in percolation models. This implies that the exponent  $\beta$  depends on the dimensionality of the system but will be the same for all three-dimensional lattices and for amorphous three-dimensional systems such as that involved in firn closure. (The exponents will also be the same for the so-called 'site' and 'bond' problems<sup>8-10</sup>, which is why the neutral terms 'step' and 'element' were used above.)

As a layered system the firn should be regarded as being two-dimensional. However, it is only very near  $p_c$  that the correlations become long enough to detect the essential two-dimensional nature so that the  $\beta$  for two-dimensional systems should apply. Further away from  $p_c$  the shorter-range correlations see an essentially three-dimensional structure. In the firn system the crossover<sup>14</sup> from  $\beta_{2D} = 5/36$  (conjectured<sup>15,16</sup>) to  $\beta_{3D} \approx 0.454$  (series estimate<sup>17</sup>) should be so close to  $p_c$  as to be undetectable. The remaining discussion will assume a purely three-dimensional description.

To model firn closure using percolation, the elements in the infinite cluster are those that are still connected to the atmosphere. If we assume that the channel closure probability,  $p$ , increases smoothly with age  $z$ , then we can consider the percolation probability as a function of  $z$  without changing the critical exponents and there will be a critical delay time,  $z_c$ , at which point all gas has been trapped.  $P(z)$  represents the proportion of gas-containing elements not trapped at time  $z$  after deposition so  $1 - P(z)$  represents the cumulative proportion trapped. The incremental trapping function  $R(z)$  is thus

$$R(z) = -\frac{\partial P(z)}{\partial z} \sim (z_c - z)^{\beta-1} \quad \text{as } z \rightarrow z_c^- \quad (3)$$

Schwander and Stauffer<sup>7</sup> measured the bubble volume of trapped bubbles as a function of depth. In the percolation model the proportion trapped is  $1 - P$ , which is plotted schematically as the solid line in Fig. 1. To convert this to bubble volume, a depth-dependent factor must be included to allow for the fact that the bubbles are subject to compression once trapped. The result is shown schematically as the dashed line in Fig. 1; the range of values observed by Schwander and Stauffer is shown by the shaded region. As the general theoretical results do not determine the specific relation between the measured ice density and the bond closure, both the horizontal and vertical scales on Fig. 1 have been linearly related to those used by Schwander and Stauffer<sup>7</sup> so as to match the points of 50% and 100% trapping. The percolation model adequately describes the geometrical aspects of bubble trapping, but the assumption of proportionality between  $z$  and the compression factor is inadequate.

Both the percolation model and the measurements by Schwander and Stauffer<sup>7</sup> determine the time of bubble trapping relative to the time of snow deposition and do not directly determine the age of the air that is trapped. However, measurements of <sup>39</sup>Ar indicate that air in the 'open' volume is well mixed throughout the firn<sup>18</sup>, so that the trapping time distribution should be close to the age distribution.

For use in solving equation (1), the percolation model will require an empirical calibration using data such as that plotted by Schwander and Stauffer<sup>7</sup> even if only to determine the amplitude of the singularity. Direct theoretical calculation of  $P(z)$  seems impractical mathematically since even highly structured lattice percolation problems do not have closed form solutions. Even an approximate calculation of  $P(z)$  would require more detail of the firn structure than is readily available.

Given the singular nature of  $R(z)$ , it is of interest to note that the ice-core deconvolution problem is less poorly posed than many typical geochemical inversion problems. Equation (1) becomes

$$q(z) \sim \int_{z-z_c}^z (z_c - z + z')^{\beta-1} c(z') dz', \text{ for } z > z_c \quad (4a)$$

or putting  $t = z_c - z$ ,  $t' = -z'$

$$q(z_c - t) \propto \int_{t-z_c}^t (t - t')^{\beta-1} c(-t') dt' \quad (4b)$$

Apart from the variable lower limit (which has little influence on the integral) this is proportional to the expression given by Ross<sup>19</sup> for defining a  $\beta$ -fold integration of  $c(-t)$  for non-integer  $\beta$ . The inverse operation of obtaining  $c$  from  $q$  corresponds to the operation denoted by

$$c(t) \propto \frac{d^\beta}{dt^\beta} q(t) \quad (5)$$

Anderssen and de Hoog<sup>20</sup> have characterized the numerical difficulty of unstable inverse problems as being given by the

equivalent order of differentiation when this is applicable. Most source deduction problems in geochemistry are essentially equivalent in difficulty to a single numerical differentiation since observed concentrations represent the integrated effects of sources. If the percolation model is valid, the deconvolution of bubble concentrations in ice cores is formally equivalent to about a 0.454th-order differentiation and so is less poorly posed than typical source deduction problems in geochemistry. Thus the resolution which can be achieved for the reconstruction of  $c$  should be somewhat better than the time period over which the firn closes.

Since submission of this manuscript we have learnt that the bubble trapping problem has also been considered in terms of the percolation model by B. Stauffer, J. Schwander and H. Oeschger (unpublished data). They performed simulations using a regular lattice approximation and concentrated on determining the critical point rather than discussing the critical exponent.

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## Prokaryotic and eukaryotic microfossils from a Proterozoic/Phanerozoic transition in China

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Cherts of the Doushantuo Formation in the Yangtze Gorges, South China, contain a superbly preserved heterogeneous assemblage of bacteria, cyanobacteria, planktonic algae, submillimetre-sized burrows and problematica. The microbiota has a bearing on defining the Precambrian-Cambrian boundary. *Baltisphaeridium* and associated spinose microfossils in the chert suggest an early Cambrian age whereas other acritarchs, vendotaenids and pretrilobite small shelly fossils from the conformably overlying the Dengying Formation, indicate a late Proterozoic age for the Doushantuo. This mixed benthic-planktonic assemblage preserved in silicified, laminated, microbial mat-like fabrics, provides a unique sample of diverse microbiotic elements existing at the dawn of the Phanerozoic and records the earliest evidence of meiofauna.

The fossil record of the latest Proterozoic is best known from Ediacaran and allied soft-bodied animals<sup>1,2</sup>, acritarchs<sup>3</sup> and stromatolites<sup>4</sup>. This record, though meagre, is fascinating, for it

was during this interval that animals originated and began to diversify<sup>5</sup>. Much of our understanding of pre-700-Myr-old palaeobiology has been based on interpreting fossils preserved in chert<sup>6</sup>; unfortunately such occurrences are not well known from post-700-Myr old deposits.

The Doushantuo Formation in the Yangtze Gorges, China, conventionally viewed as late Proterozoic<sup>7,8</sup>, contains cherts with well-preserved, abundant and diverse microfossils such as coccoid and filamentous cyanobacteria, bacteria, large spiny organic-walled microfossils, problematic tubular microfossils, other problematica and submillimetre-sized backfilled burrows. In addition to a benthic mat biota, the cherts preserve elements of the phytoplankton that lived in the overlying water column.

The Doushantuo Formation is a dark brown to grey-black dolomite with chert, interbedded with shale. It unconformably overlies tillite of the Nantuo Formation and is conformably overlain by dolomite and limestone of the Dengying Formation<sup>9</sup> (Figs 1, 2).

The Yangtze Gorges region contains the type section for the Chinese Sinian System<sup>10</sup> (composed, in ascending order, of the



Fig. 1 Index maps showing location of measured sections.



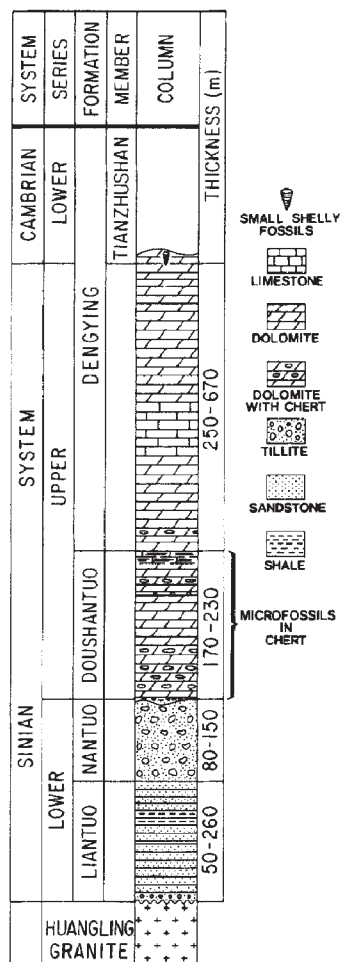


Fig. 2 Generalized stratigraphical column of Sinian System in the Yangtze Gorges, Yichang County, Hubei, China.

Liantuo, Nantuo, Doushantuo and Dengying formations). It would be a potential candidate for any global stratotype section that may be established for a new geological system to embrace the pre-shelly fossil record during which metazoans appeared<sup>11</sup>. Here the Sinian is a thick succession with such lithological and palaeontological curiosities as tillite, phosphorite, acritarchs, vendotaenid algae, stromatolites, oncolites, microfossiliferous chert, putative Ediacaran-type metazoans, sponge-like spicules, trace fossils and is conformably overlain by the small shelly fossil-bearing Tianzhushan member (upper Dengying Formation), regarded as signalling the base of the Cambrian<sup>9,12</sup>.

Within this stratigraphical framework, the Doushantuo is usually taken to be Late Sinian (Vendian or Ediacarian), probably <700 Myr-old. Although their reliability may be questioned, Rb-Sr whole rock isochron ages from the top of the formation yield  $700 \pm 5$  and  $691 \pm 29$  Myr (ref. 13). Other potential radiometric age constraints include an ion-probe U-Pb age of  $740 \pm 16$  Myr on zircons in tuff from the Lower Sinian Liantuo Formation<sup>13</sup>, and ages of  $806 \pm 6$  Myr (Rb-Sr isochron) and  $823 \pm 7$  Myr (K-Ar on biotite) from the crystalline basement underlying the Liantuo<sup>13</sup>. Shale from the upper part of the Tianzhushan member (Dengying Formation) has an inferred isotopic age of  $610 \pm 10$  Myr based on whole rock Rb-Sr isochron analyses<sup>14</sup>. Palaeontologically, acritarchs from acid residues of Doushantuo clastic rocks have been interpreted to indicate a late Proterozoic age (<700 Myr)<sup>7,15</sup>. The overlying Dengying Formation's palaeontology (vendotaenids from the lower part<sup>7</sup>; pre-Cambrian acritarchs from shale<sup>7,9</sup>, and small shelly fossils of Meichucunian or Tommotian character at the top<sup>7</sup>) also indicates a pre-Cambrian age for the Doushantuo. Following guidelines set forth by the International Union of Geological Sciences/International Geological Correlation Program Project

29 Working Group on the Precambrian-Cambrian Boundary<sup>16</sup>, the small shelly fossils appearing in the Tianzhushan member (Dengying Formation; Fig. 2) would define the base of the Cambrian. Hence, until now, the overall palaeontology, stratigraphy, isotope age data and correlations with other areas suggested a late Proterozoic age for the Doushantuo.

Previously reported fossils from the Doushantuo Formation include stromatolites<sup>17</sup>, acritarchs<sup>15</sup> and chitinozoans<sup>17</sup>. Cyanobacteria<sup>18-20</sup> and spiny microfossils<sup>21,22</sup> have been described from chert and sponge-like spicules from dolomite<sup>17</sup>. No megascopic algal, metazoan trace or body fossils have yet been found in the Doushantuo.

The microfossils that we describe here were detected in petrographic thin sections of chert nodules in dolomite from two measured sections of the Doushantuo Formation; one ~150 m east of Wangfenggang Village, the other ~700 m south-southeast of Tianjiayuanzi Village, Yichang County, Hubei, China (Fig. 1). Of the coccoidal microfossils detected, solitary specimens are rare; the more abundant pluricellular varieties are represented by such Proterozoic taxa as *Myxococcoides*, *Palaeoanacystis* and *Tetraphycus*. Among the new taxa present are a *Merismopedia*-like pluricellular form with tightly packed cubic cells and an aggregate of small <1-μm diameter cells that might represent fossilized bacteria. In general, most of the coccoidal morphs resemble cyanobacteria; however, although diverse, the coccoids are only moderately abundant when compared with filaments and large spinose morphs.

The benthic component of the microbiota is dominated by filamentous fossils that resemble cyanobacteria. Bundles, 50-400 μm across, of filaments, 5-45 μm in diameter, are common (Fig. 3a) and many superficially resemble the extant multi-trichomous oscillatoriacean *Microcoleus*. Numerous single filaments, ranging from <1 to as much as 100 μm in diameter, and representing several taxa, occur as solitary strands and in mat-like arrangements. Rare examples of an *Obruchevella*-like fossil have been found. Most filaments resemble the empty sheaths of cyanobacteria although others may be bacteria and algae. Preservation at times is so good that a trichome can be found preserved within its sheath (Fig. 3d). No heterocystous or branched filaments have been detected.

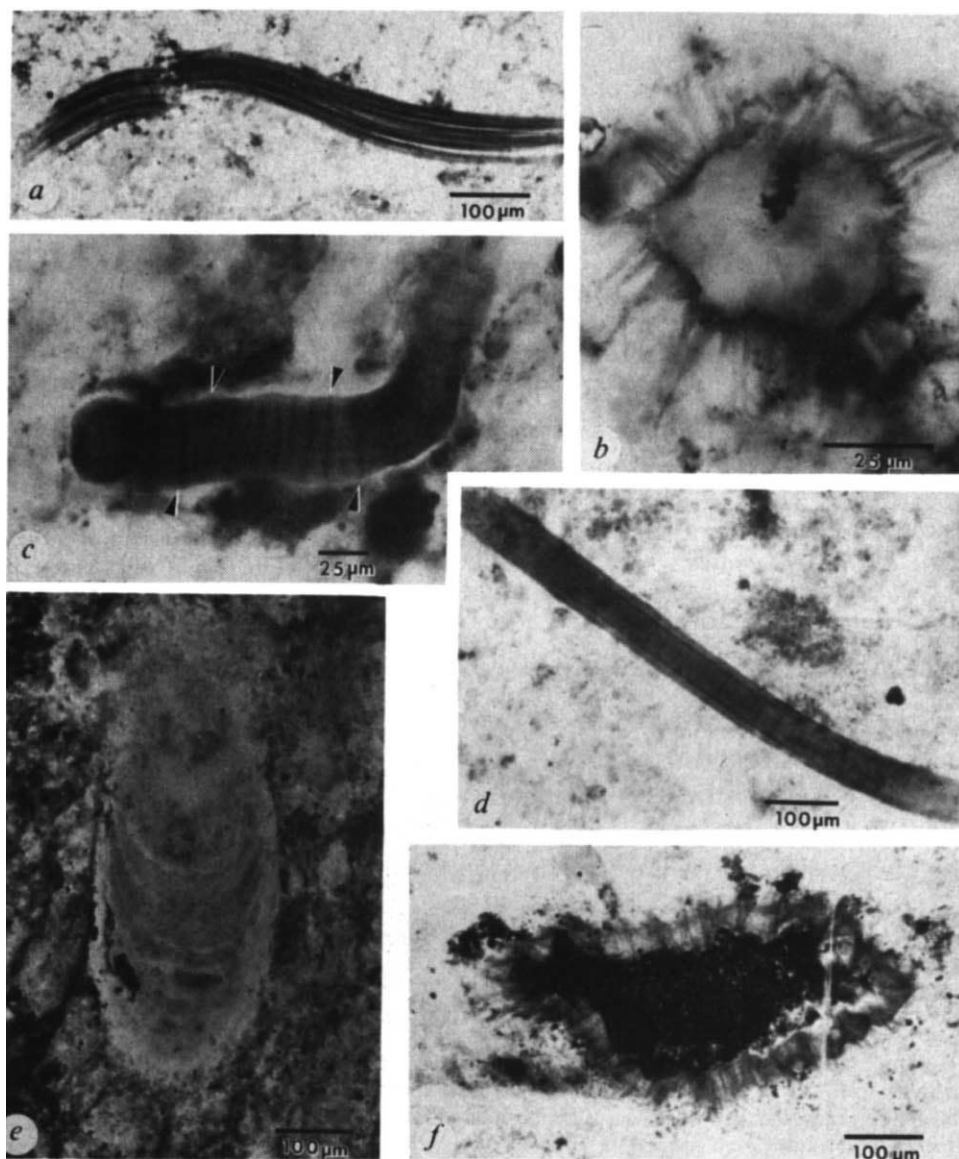
Cyanobacteria-like fossil diversity is high; >20 species are present. These microfossils occur in rudimentary, stratiform stromatolite-like microstructures not too unlike those seen in Proterozoic microfossiliferous stromatolites<sup>23</sup>. In some instances, this rudimentary lamination is disrupted in a manner suggesting churning of the sediment by organisms that did not leave clear, well-defined burrows.

Planktonic microfossils include a variety of morphotypes. Among the most conspicuous and abundant plankters found in thin section are diverse spinose vesicles, ranging from 30 to 1,200 μm across. Important among these are the spiny acritarchs resembling *Baltisphaeridium* (Fig. 3b) hitherto known only from early Cambrian and younger rocks<sup>24,25</sup>.

The Doushantuo also contains abundant and diverse, morphologically-complex microscopic fossils of uncertain systematic position. First, there are robust, multilayered and partitioned tubes (Fig. 3c). These fossils, 40-100 μm in diameter, occur in variable abundance in numerous thin sections from several sites. At first glance, these tubes resemble large, lamellated, oscillatoriacean cyanobacterial sheaths. However, oscillatoriaceans and other filamentous cyanobacteria, bacteria, algae, and fungi are not known to possess this type of architecture. Superficially they resemble miniaturized coelomates; however, the small size and apparent partitioning of the innermost tube (which would be equivalent to the gut) make this affinity unlikely. This morph might represent an evolutionary novelty in the prokaryotic or eukaryotic line for which there are no modern analogues.

Also, large, 400-900-μm wide and 750-1,500-μm long, morphologically complex, brown coloured vesicles (Fig. 3f) have been found in many thin sections from several sites. The fossils are characterized by a tripartate organization consisting of: (1)

**Fig. 3** Microfossils from the Doushantuo Formation. All photomicrographs are from petrographic thin sections housed in the Preston Cloud Research Laboratory. *a*, Bundle of filaments showing possible multitrichomous habit. Sheath is not evident. *b*, *Baltisphaeridium* spiny microfossil; note the closed, tapered ends of the spines. *c*, Partitioned, concentrically-layered tube. Partitions, indicated by arrows, cut the layered cylinder. It is uncertain even to what kingdom this fossil belongs. *d*, Cyanobacterium with trichome preserved within lamellated sheath. The outer sheath is not partitioned and the inner trichome is not layered. *e*, Backfilled burrow. This burrow and others like it (preserved in chert), pre-date previously known bona fide burrows from the Sinian of China. *f*, Complexly organized protistan showing outer layered organic mantle with tube clusters, boundary layer, and interior with particulate contents.



the vesicle interior that typically contains amorphous black particulate organic and pyritic matter and occasionally folded membrane-like material; (2) a well-defined boundary layer or wall that separates an external organic covering from the vesicle interior; and (3) an outer organic covering or mantle that is occasionally layered, as seen in Fig. 3f. This organic covering contains clusters of from 3 to 8 small, hollow tubes, each 1–3  $\mu\text{m}$  in diameter. The tubes have closed, tapered ends. Where attached to the boundary, the tubes open to the vesicle's interior. The tubes, wall structure, interior and overall organization of these microfossils are unlike that of previously described spinose acritarchs, although some similarities exist with the Proterozoic morph *Trachyhystrichosphaera*<sup>26</sup>. Superficially they also resemble certain protozoan sporocysts like those of gregarines. The size and complexity of these fossils indicate some kind of eukaryote, probably a protistan. Again the possibility exists that these fossils have no modern analogues.

Structures resembling backfilled burrows, 150–250  $\mu\text{m}$  across (Fig. 3e), are known from two collecting sites. Longitudinal and cross-sections of these burrows are found oriented both parallel to, and oblique to, lamination in chert nodules. Although rare (four examples known) and in the case of Fig. 3e composed of chalcedony, they probably represent some worm-like meiofaunal animal that peristaltically moved through sediment. These structures cannot be interpreted as laminated constructional features like microstromatolites for the layering is concave down and disrupts chert matrix laminae. The presence of such

submillimetre-sized, backfilled burrows in the Doushantuo suggests meiofaunal activity and, if the age of the Doushantuo proves to be late Proterozoic, make them among the oldest burrows known.

Clearly, the discovery of *Baltisphaeridium* and related spinose vesicles in Doushantuo cherts raises questions, not only about the age of the Doushantuo, but also concerning criteria and reliability of criteria used to define the Precambrian–Cambrian boundary.

This precise age controversy aside, the exceptional preservation of the abundant and diverse prokaryotic and eukaryotic fossils in the Doushantuo cherts provide a unique microscopic window on a time during the early stages of animal evolution and the waning of stromatolite-building microbial mat communities<sup>27</sup>. These new data must be considered when defining one of the major boundaries of the geological time scale. The robust nature of many of the cyanobacteria (large size, thick sheaths) as well as some of the problematic morphs may have been an attempt by mat-forming organisms to cope with newly evolved 'meiofaunal' and macrofaunal benthic eukaryotic heterotrophs that ultimately overwhelmed the stromatolite-building biocenoses: an extraordinarily successful biocenose that had flourished for hundreds of millions of years.

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## Energy transfer at the surface of clays and protection of pesticides from photodegradation

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Certain potential pesticides are of limited use because of their sensitivity to sunlight. Previous attempts to overcome this limitation have been unsatisfactory because of impairment of insecticidal efficiency or unwanted toxic properties. The photosensitivity results from the balance between the processes, such as energy transfer and chemical reaction, by which photoexcited molecules can release excess energy and return to the ground state. The efficiency of the various energy transfer mechanisms<sup>1-5</sup> depends, in some cases, on the specific spectroscopic characteristics of donor and acceptor molecules, the distance between them and their relative orientations. We present here an approach aimed at protecting compounds of agricultural interest (for example, pesticides) by exploiting the unique surface properties of certain clays in order to build systems in which fast energy transfer occurs before photodegradation starts.

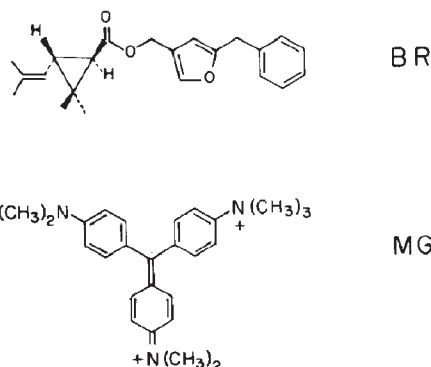
Clays, such as montmorillonite, exhibit outstanding adsorption properties due to their high specific surface areas and high cation-exchange capacities<sup>6</sup>. As organic molecules adsorbed on the clay surface are oriented<sup>7</sup>, their intermolecular distances and orientations might fulfil the requirements for an efficient energy transfer process. Measurements of energy transfer on clay surfaces have been described for certain fluorescent cations<sup>8-10</sup>. However, occurrence of such processes between adsorbed non-fluorescent photolabile organic molecules has not been reported.

A suitable target for study of photostabilization is the synthetic non-halogenated pyrethroid bioresmethrin (BR) (5-benzyl-3-furylmethyl) (1R)-trans-chrysanthemate, a powerful contact insecticide effective against a wide range of insect pests and one of the safest pesticides (its acute oral LD<sub>50</sub> (50% lethal dose) for rats is 8 g per kg). Despite these advantages, bioresmethrin is not used in agriculture owing to its rapid photodecomposition.

It was found that chemical modification of the pyrethroid structure leads, in some cases, to more stable pesticides<sup>11-16</sup>. Attempts have been made to stabilize the photolabile pyrethroids using various additives (antioxidants and UV-screensers)<sup>17-22</sup>. Recently, the photostabilization of cyphenothrin by dinitroanilines at certain relative concentration ratios has been attributed to possible exciplex or charge transfer quenching<sup>22</sup>.

We have achieved stabilization of BR by adsorbing it on montmorillonite (Clay) together with the divalent cation methyl green (MG). Figure 1 shows the results of a typical bioassay using adults of the flour beetle *Tribolium castaneum*. Control samples of free BR exposed to sunlight completely lost their biological activity in <2 h, whereas samples prepared according to our method were still active after several days of exposure to sunlight. BR adsorbed in a complex with 0.2 mmol of MG per g clay was photochemically more stable than BR in a complex with a higher level of dye (0.4 mmol g<sup>-1</sup>). This indicates that the stabilization effect is due to specific interactions between the two compounds at the surface of the clay rather than to merely a UV-screening effect.

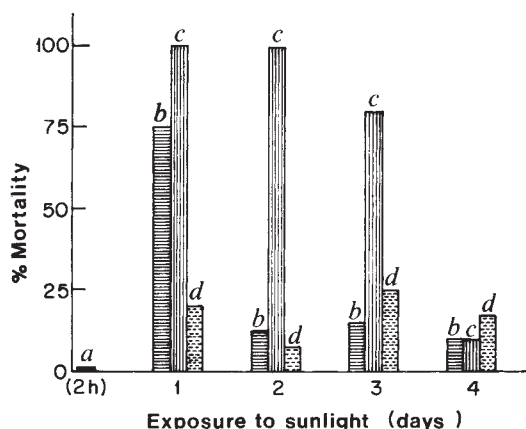
Some details of these interactions at the molecular level can be obtained using Fourier transform IR (FTIR) spectroscopy (Fig. 2). A comparison of spectrum *a* (free BR) with spectrum *b* (BR adsorbed on clay) reveals that the stretching frequency of the C=O bond shifts from 1,722 to 1,709 cm<sup>-1</sup> on adsorption.



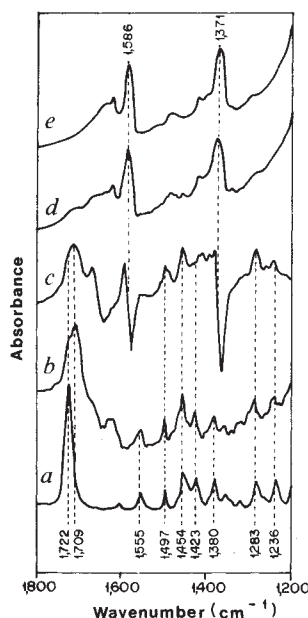
The position of this band in spectrum *c* (BR adsorbed in the presence of MG) clearly shows that the BR molecules in the Clay-MG-BR complex are adsorbed in a similar manner as in the case of Clay-BR. Minor changes in the strong absorption bands of MG at 1,568 and 1,371 cm<sup>-1</sup> are also induced by the co-adsorption of BR. These changes, although difficult to observe in the measured spectra (Fig. 2*d, e*), are evident in the difference spectrum (Fig. 2*c*). In addition, co-adsorption of MG and BR induces broadening of the 1,497 cm<sup>-1</sup> peak of BR. The fact that vibrational frequencies of one organic molecule are shifted due to the co-adsorption of another one (as detected by the differential FTIR technique) indicates that short-distance intermolecular interactions exist between the molecules of these substances on the clay surface. A change in concentration of the adsorbed molecules results in a modification of the intermolecular distances and/or relative orientations in a manner which evidently affects the efficiency of the photostabilization effect (Fig. 1).

The method we propose for protecting pesticides from photodegradation thus consists of adsorbing the photolabile agrochemical to the surface of a clay to which another organic chromophore has been attached. This second organic chromophore may be selected according to its spectroscopic, geometric and charge characteristics, depending on the type of clay to be used and the pesticide to be protected. If the clay is a smectite, such as montmorillonite, with a negatively charged surface, a cationic protective compound may be used. As a result of its adsorption to the clay, the charge at the adsorption site is neutralized and the surface might become hydrophobic (depending on the size of the organic cation). If a non-ionic pesticide molecule is added to this system, it will be adsorbed at these hydrophobic sites or, alternatively, it might be attached

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**Fig. 1** Bioassay to evaluate protection of bioresmethrin from photodegradation. *a*, Unprotected bioresmethrin; *b*–*d*, montmorillonite(Clay)–methyl green(MG)–bioresmethrin(BR) complexes with different MG loading. The concentration of BR in all three cases was 0.2 mmol per g clay. Concentrations of MG, relative to clay, were: *b*, 0.1 mmol g<sup>-1</sup>; *c*, 0.2 mmol g<sup>-1</sup>; *d*, 0.4 mmol g<sup>-1</sup>. Clay–MG–BR complexes were prepared as follows: aliquots of a 10<sup>-3</sup> M aqueous solution of MG were added dropwise to a 0.5% aqueous suspension of Na-montmorillonite (SWy-1 from the Clay Minerals Society repository). After separation by centrifugation at 16,000g, the precipitate was washed three times with distilled water, freeze-dried and ground to <50 µm. The appropriate volume of a 10<sup>-2</sup> M solution of BR in hexane was added to this powder and the solvent was evaporated under reduced pressure. For bioassays, 10 mg of the complex was deposited on the bottom of Petri dishes (5-cm diameter) and exposed to sunlight for various intervals of time; 2-week-old *Tribolium castaneum* (CSbb strain) adults were used. After irradiation, 20 insects were transferred from stock cultures to the Petri dishes and the percentage mortality was recorded. Irradiated controls of Clay–BR complexes (BR adsorbed to montmorillonite in the absence of MG) gave 0% mortality after 4 h of exposure to sunlight. In all cases controls protected from light gave 100% mortality.



**Fig. 2** Fourier transform IR absorption spectra of: *a*, bioresmethrin (BR); *b*, BR adsorbed on montmorillonite. The latter spectrum was obtained by subtracting the spectrum of the clay from that of the complex Clay–BR (none of which are shown). *c*, BR adsorbed in the complex Clay–MG–BR, obtained by subtraction of spectra *e* and *d*. 'Negative' peaks just below 1,586 and 1,371 cm<sup>-1</sup> result from small shifts in the MG transitions at these positions due to co-adsorption of BR. *d*, Clay–MG–BR, with 0.2 mmol of each of MG and BR per g clay; *e*, Clay–MG (0.2 mmol MG per g clay). All spectra were measured in KBr pellets using a Nicolet MX-S FTIR spectrophotometer.

to the clay surface either directly or through interaction with adsorbed inorganic cations and/or interlayer water molecules. The actual mechanism will depend largely on the structure of the pesticide molecule (nature of functional groups) and on the extent of coverage of clay surface by the organic cation. Following photoexcitation of pesticide molecules, an efficient energy transfer between them and neighbouring organic cations might take place at a high rate, thus preventing the photochemical decomposition of the bioactive compound. In such a system, the protective compound is linked to the clay surface by strong coulombic bonds, whereas the pesticide molecules are retained by much weaker bonds and can be easily detached from the surface, as required for insecticidal activity.

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## Patterns of family extinction depend on definition and geological timescale

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The recent claim<sup>1</sup> of an approximate 26-Myr periodicity in the pattern of mass species extinction over the past 250 Myr has already stimulated much astrophysical speculation and debate on causal mechanisms<sup>2–13</sup>. However, as shown here, the evidence for that claim is strongly contingent on arbitrary decisions concerning the absolute dating of stratigraphical boundaries<sup>14</sup>, the culling of the database and the definition of what is mass extinction as opposed to background extinction. This evidence becomes insufficient under other plausible geological timescales and other acceptable definitions of mass extinction. Analysis of the non-culled database shows that the reliability of identification of mass extinctions and their timing is at present extremely limited. It also suggests that the apparent periodicity of mass extinctions results from stochastic processes.

The database for recent studies on global patterns of diversity and extinction usually consists of Sepkoski's compendium of stratigraphical ranges of all marine animal families in the Phanerozoic<sup>15</sup>. The stratigraphical resolution is to the level of universally recognizable units called stages (some of them are indeed stages, others are clusters of stages or even series). This compendium is taken at its face value, although the earlier



**Table 1** Late Permian to Pleistocene maxima of marine animal family extinction, depending on geological timescale (H-H3 and O-O3) and definition of the maxima (A-F)

|    | Permian                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         | Triassic | Jurassic | Cretaceous | Tertiary | Quaternary |
|----|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------|----------|------------|----------|------------|
|    | Guadelupian<br>Dzhulfian<br>Induan<br>Olenekian<br>Anisian<br>Ladinian<br>Carnian<br>Norian<br>Rhaetian<br>Hettangian<br>Sinemurian<br>Pliensbachian<br>Toarcian<br>Bajocian<br>Bathonian<br>Callovian<br>Oxfordian<br>Kimmeridgian<br>Tithonian<br>Berriasian<br>Valanginian<br>Hauterivian<br>Barremian<br>Aptian<br>Albian<br>Cenomanian<br>Turonian<br>Coniacian<br>Santonian<br>Campanian<br>Maestrichtian<br>Danian<br>Late Palaeocene<br>Early Eocene<br>Middle Eocene<br>Late Eocene<br>Early Oligocene<br>Late Oligocene<br>Early Miocene<br>Middle Miocene<br>Late Miocene<br>Pliocene<br>Pleistocene |          |          |            |          |            |
| A  | *                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               | o        | *        | *          | *        | o          |
| B  | *                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               | *        | *        | *          | *        | o          |
| C  | *                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               | *        | *        | *          | *        | o          |
| D  | *                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               | *        | *        | *          | *        | o          |
| E  | *                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               | *        | *        | *          | *        | o          |
| H  | *                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               | *        | *        | *          | *        | o          |
| H1 | *                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               | *        | *        | *          | *        | o          |
| H2 | *                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               | *        | *        | *          | *        | o          |
| H3 | *                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               | *        | *        | *          | *        | o          |
| O  | *                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               | *        | *        | *          | *        | o          |
| O1 | *                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               | *        | *        | *          | *        | o          |
| O2 | *                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               | *        | *        | *          | *        | o          |
| O3 | *                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               | *        | *        | *          | *        | o          |
| F  | *                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               | *        | *        | *          | *        | o          |
| H  | *                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               | *        | *        | *          | *        | o          |
| H1 | *                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               | *        | *        | *          | *        | o          |
| H2 | *                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               | *        | *        | *          | *        | o          |
| H3 | *                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               | *        | *        | *          | *        | o          |
| O  | *                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               | *        | *        | *          | *        | o          |
| O1 | *                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               | *        | *        | *          | *        | o          |
| O2 | *                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               | *        | *        | *          | *        | o          |
| O3 | *                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               | *        | *        | *          | *        | o          |

Asterisks denote major maxima, circles denote minor, perhaps insignificant maxima. A, Probabilistic rate of extinction per stage, culled data set, maxima defined as local peaks; B, number of family extinctions per stage, complete data set, maxima defined as local peaks; C, probabilistic rate of extinction per stage, complete data set, maxima defined as local peaks; D, probabilistic rate of extinction per stage, complete data set, maxima defined as points more than one standard deviation above the regression line (the same maxima obtain for the number of family extinctions per stage); E, number of family extinctions per Myr, complete data set, maxima defined as local peaks (H, timescale given by Harland *et al.*<sup>33</sup>; H1, H2, H3, timescales randomized within the limits of analytical error of absolute dates given by Harland *et al.*; O, timescale given by Odin<sup>32</sup>; O1, O2, O3, timescales randomized within the limits of analytical error of absolute dates given by Odin); F, probabilistic rate of extinction per Myr, complete data set, maxima defined as local peaks (timescales as for E).

demonstration by Raup<sup>16,17</sup> and others<sup>18-20</sup> that these kinds of data are strongly biased has never been refuted. The data are at the family level of taxonomic hierarchy, and the resultant patterns may not agree with the patterns at the species level<sup>21</sup>. For instance, the Toarcian maximum of species extinction<sup>22-24</sup> is unrecognizable at the family level, whereas the late Eocene maximum of family extinction does not show up at the species level<sup>25-28</sup>. Therefore, the following analysis is, for the sake of consistency, confined to the family level.

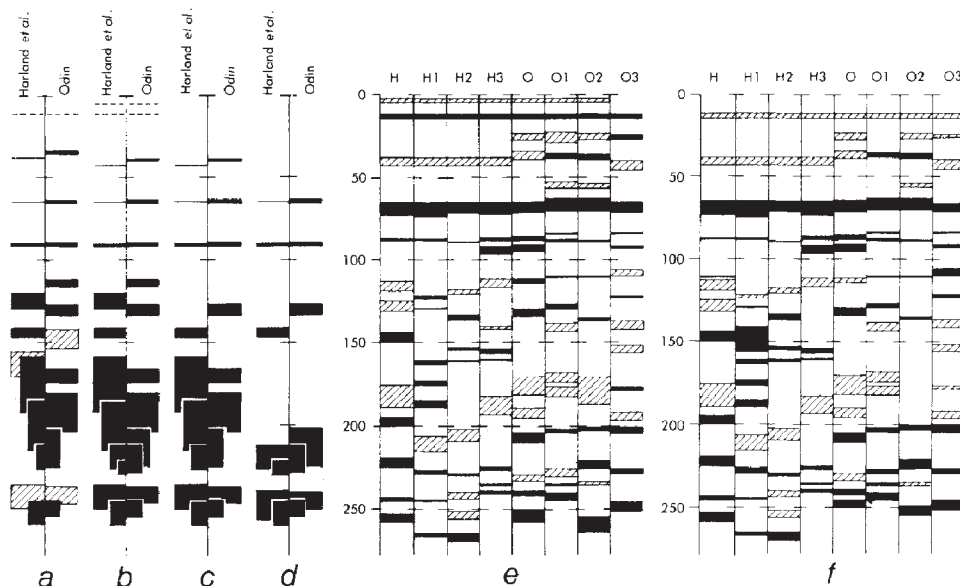
The database analysed for periodicity in mass extinction<sup>1</sup> is only a subset of Sepkoski's compendium. This subset is defined by exclusion of extant families and families with imprecise or questionable stratigraphical ranges. The time frame is the latest Permian (Dzhulfian) to middle Miocene. The data culling was intended to remove the noise and the pull of the Recent<sup>16</sup>, but it results in data reliability being much lower in the Cenozoic than in the Mesozoic. For instance, the middle Miocene demise of 5 families becomes a possible mass extinction and the late Eocene demise of 10 families is a very pronounced event, whereas 19 families going extinct in the Campanian represents a period of low extinction and the late Permian extinction involves hundreds of families. Clearly, the alleged mass extinction events are incompatible with each other. Furthermore, the removal of extant families causes some extinction peaks to shift; probability of family extinction is higher in the late Eocene than in the middle Eocene only because the family number, that is, denominator, is artificially decreased in the former. The data culling also results in arbitrariness, for there is no criterion to

determine which stratigraphical ranges are unquestionable. This must be an arbitrary decision because each range may be extended in either direction by new discoveries (probabilistic methods<sup>29,30</sup> are useless because neither family encounter frequency nor sequence in independent samples can be analysed). The time frame of Raup and Sepkoski's analysis arbitrarily cuts off the Guadelupian (extinction rate and probability higher than in the Dzhulfian) as well as the late Miocene (five family extinctions, as in the middle Miocene) and Pliocene (seven family extinctions). Had the Guadelupian and Pliocene, rather than Dzhulfian and middle Miocene, been accepted as mass extinctions, the 26-Myr periodicity would have to be rejected.

Hence, the following analysis is based on all Guadelupian to Recent taxa recorded in Sepkoski's compendium. Family extinctions with stratigraphical resolution to the series or system level are assigned proportionately to the stages, a procedure which accentuates maxima of extinction. Nevertheless, the culled data set analysed by Raup and Sepkoski shows eight maxima with extinction probability exceeding 0.1 (cutoff level for mass extinctions at the family level<sup>2,31</sup>), whereas the complete data set gives only two events of this magnitude (Guadelupian, Maestrichtian) and only four additional events exceeding the 0.05 level (Olenekian, Norian, Tithonian, Cenomanian). This difference clearly shows the impact of data culling by Raup and Sepkoski.

Analysis of the timing of geological or palaeontological events obviously must depend on the geological timescale. There is much uncertainty in absolute dating of stratigraphical bound-

**Fig. 1** Time distribution of maxima of family extinction, depending on definition and geological timescale (see Table 1 for explanation and event identification); vertical scale in Myr. Mass extinction events occurred somewhere within time ranges given by the black bars; hatched bars (or dashed lines) denote minor, perhaps insignificant, maxima.



daries. Two recent timescales<sup>32,33</sup> differ by as much as 10–14 Myr for many stratigraphical boundaries in the Mesozoic. This difference might increase to over 20 Myr if the analytical errors of absolute dates, as estimated by the authors of the two scales, run in opposite directions. (The timescale uncertainty is thus not much less than in the post-Cambrian Palaeozoic, where no 26-Myr periodicity in mass extinctions has been discovered<sup>1</sup>.) The correlation is only moderate between the two timescales and its statistical significance is largely due to the generally good dating, and hence agreement between the timescales, for the late Cretaceous and Cenozoic. This correlation, however, dramatically decreases for randomized modifications of the timescales, with stage durations determined at random within the limits of analytical error of the absolute dating as given by the authors of the timescales. Such a randomization assumes that, contrary to the timescale of Harland *et al.*<sup>33</sup> accepted by Raup and Sepkoski, stratigraphical stages vary in duration. This assumption, however, is strongly corroborated by the evidence from, for instance, late Cretaceous stages ranging from 1 Myr (Coniacian) to 15.5 Myr (Albian).

Analysis of the timing of mass extinctions naturally depends on the choice made from a variety of criteria for distinguishing between mass extinction and background extinction. This is not a trivial matter, for, as noted by Raup *et al.*<sup>34</sup>, “it is entirely possible that the initial assumption . . . that background extinction and mass extinction constitute two discrete populations, may not be correct”. Rate or probability of extinction can be taken as extinction metrics. If extinctions are assumed to occur episodically rather than continually, the use of rate or probability per stratigraphical stage is reasonable. This is an *ad hoc* assumption but it may be substantiated by the evidence for catastrophic physical events (for example, extraterrestrial impacts) as the causes of mass extinction and by the absence of correlation between extinction and stage duration<sup>1</sup>. There is, in fact, strong evidence that the terminal Maestrichtian extinction is associated with<sup>35–41</sup>, though not necessarily caused by<sup>42–46</sup>, a major physical catastrophe, and only disputable evidence for coincidence of the late Eocene extinction peak with a physical catastrophic event<sup>26,47,48</sup>. However, evidence for association of other alleged mass extinctions with major catastrophes is largely absent<sup>46</sup>. On the other hand, the absence of correlation between extinction and stage duration may be caused by the fallacy of equal stage durations; besides, it is irrelevant to individual events. If the assumption that extinctions occur episodically is rejected, extinction rate or probability per Myr should be applied as the appropriate extinction metric, as in the analyses of extinction intensity through geological time<sup>49,50</sup>.

Mass extinctions may be defined as peaks of the curve<sup>1</sup>. An arbitrary level of extinction metric may, then, be set to distinguish between true and spurious peaks caused by minor and

inevitable fluctuations in background extinction<sup>2,31</sup>. Such a definition is appropriate for extinction metrics normalized for time, although it ignores the possibility of prolonged periods of unusually high extinction. As judged after Sepkoski's data<sup>15</sup>, the latter may be the case with (Givetian?–) Frasnian–Famennian, Guadalupian–Dzhulfian and Carnian–Norian–Rhaetian. On the other hand, definition of mass extinctions as peaks of the curve rules out the possibility that two, or even more, consecutive stages were periods of mass extinction. Yet instantaneous mass extinction events may well be separated by one stage only. This may be an alternative interpretation of consistently high extinction in the late Devonian, late Permian and late Triassic if each stage was taken to represent an individual event. Therefore, such a definition is inappropriate for extinction metrics per stratigraphical stage. Instead, all rates or probabilities exceeding a certain arbitrary level can be recognized as mass extinctions. Alternatively, rates or probabilities more than, say, one standard deviation above the regression line over geological time can be identified as mass extinctions. The latter definition takes into account the substantial decrease in extinction intensity through time<sup>49,50</sup>.

Table 1 shows the wide array of stages that appear as mass extinctions, depending on definition and timescale. There is some apparent regularity in the pattern of mass extinction events defined as peaks of extinction metrics not normalized for time (Table 1A–C), but this may well be pseudoperiodic behaviour of a stochastic system; when the absolute magnitude of alleged mass extinctions is taken into account in addition to their timing, such a model shows a better fit to the empirical pattern<sup>51</sup>. When the absolute magnitude of extinction peaks is disregarded, however, as in Raup and Sepkoski's analysis, such a definition causes the empirical pattern to represent only relative changes in extinction metric over relative geological time. Thus, the conditions for random walk are met. Given a strict random-walk model (0.5 probabilities of both increase and decrease in extinction metric, regardless of what happened at the preceding step), there is 0.25 probability that any particular stage represents a peak of extinction. Peaks are, then, expected to occur approximately every fourth stage, albeit with considerable variance. In fact, their actual distribution in the Phanerozoic does not significantly deviate from this expectation of random-walk model<sup>52</sup>. Given the assumption of equal stage durations for the Triassic to early Cretaceous and given also 6.2 Myr as the average stage duration in the Guadalupian to Quaternary, the 26-Myr periodicity of extinction peaks in that time interval also does not significantly deviate from expectation. (The average stage duration is considerably longer in the Palaeozoic and, therefore, 35–40-Myr periodicity can be expected for this era, which may explain the misfit between Palaeozoic and post-Palaeozoic data reported by Raup and Sepkoski<sup>1</sup>.)



On the other hand, the Pliensbachian, Bajocian, Tithonian and Hauterivian rather consistently stand out as significant events in Table 1. These stages, however, also represent peaks of family origination<sup>52</sup>, despite the absence of overall correlation between extinction and origination<sup>53</sup>. This may suggest that they were simply longer than adjacent stages; such a hypothesis is especially plausible for the Bajocian, as it also includes the Aalenian in the stratigraphical framework of Sepkoski's compendium.

There are few stages that do not appear as maxima of family extinction in Table 1, regardless of timescale and definition, although some of them might, given other randomizations of the timescale (only three randomizations were made for each of the two timescales<sup>32,33</sup> considered here). Only the Maestrichtian event stands out regardless of timescale and extinction metric. The late Permian maximum shifts from the Guadalupian to Dzhulfian under some timescales, and both of them depart strongly from the regression line of probabilistic extinction rate over time. It is a subjective matter whether the late Triassic maximum is placed in the Carnian, Norian or Rhaetian (in fact, both the Carnian and Norian strongly depart from the regression, whereas the Rhaetian was probably the shortest and, hence, gains prominence if the extinction metric is normalized for time). Under time-normalized extinction metrics, virtually each Jurassic and Cretaceous stage may be a period of mass extinction. If extinction probability per Myr is considered, there seems to be no important event during the 65 Myr of the Cenozoic (the range of variation of the extinction metric does not exceed 0.002 in the Cenozoic, whereas it is an order of magnitude wider in the late Cretaceous); and there is only one late Jurassic event throughout the Jurassic and early Cretaceous (~120 Myr), given some timescales.

When plotted against absolute time (Fig. 1), the pattern of mass extinctions becomes even more ambiguous. Even under the same definition of extinction maxima, there is much uncertainty about their timing. To overstate the case, the Pliensbachian and Bajocian events (if there were such) may be as close to each other as 5 Myr or as far apart as 40 Myr according to Harland *et al.*<sup>33</sup> (this range is somewhat smaller under the Odin timescale<sup>32</sup>). If the extinction metric is normalized for time, there is almost no invariance in time distribution of major events.

Thus, the assumption that all major mass extinction events are correctly identified and that no event is either missing from or redundant in the list<sup>2,24</sup> is absolutely unwarranted. Non-parametric testing is, therefore, appropriate. The test developed by Raup and Sepkoski<sup>1</sup> and Rampino and Stothers<sup>12</sup>, however, is very liberal, for it is insensitive to the absence of predicted events. Hence, the test is not expected to reject the claim for periodicity even if some minor peaks (for example, Olenekian, Bajocian, Callovian, Hauterivian, middle Miocene) are no longer regarded as true mass extinctions. Some extinction metrics pass that test for various cycle lengths under certain timescales (for example, events defined as in Table 1D fit the 26-Myr periodicity under the timescale of Harland *et al.* and no other timescale considered herein). Correlation to various external cycles could, then, be sought and *ad hoc* explanations given for absence of mass extinctions from where they should be (for example, absence of any early to middle Jurassic, early Cretaceous, and Cenozoic events in Table 1D). If, however, such absences are considered real, or if minor events are also taken into account, the appearance of periodicity vanishes in most cases.

It is crucial that what is a matter of interpersonal agreement is not accepted as a matter of discovery. There may be periodicity in late Permian to Quaternary mass extinctions, but we only know that there were periods (not necessarily short) of increased extinction in the late Permian, late Triassic, late Cretaceous and perhaps also late Jurassic and mid-Cretaceous. The exact timing of these events and what other events, if any, are to be considered mass extinctions, are subject to arbitrary decisions. It is those subjective decisions that result in the appearance of periodicity of mass extinctions. However, even if the objective pattern of

mass extinctions did indeed appear periodic, this might be due to combined effects of independent processes rather than to a single causal mechanism<sup>51,52</sup>. Hence, the biological and geological context of each event must be evaluated on its own.

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## Outer hair cells in the mammalian cochlea and noise-induced hearing loss

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**Hair cells in the mammalian cochlea transduce mechanical stimuli into electrical signals leading to excitation of auditory nerve fibres<sup>1</sup>. Because of their important role in hearing, these cells are a possible site for the loss of cochlear sensitivity that follows acoustic overstimulation<sup>2,3</sup>. We have recorded from inner and outer hair cells (IHC, OHC) in the guinea pig cochlea during and after exposure to intense tones. Our results show functional changes in the hair cells that may explain the origin of noise-induced hearing loss. Both populations of hair cells show a reduction in amplitude and an increase in the symmetry of their acoustically evoked receptor potentials. In addition, the OHCs also suffer a sustained**

depolarization of the membrane potential. Significantly, the membrane and receptor potentials of the OHCs recover in parallel with cochlear sensitivity as measured by the IHC receptor potential amplitude and the auditory nerve threshold. Current theories of acoustic transduction<sup>4-6</sup> suggest that the mechanical input to IHCs may be regulated by the OHCs. Consequently, the modified function of OHCs after acoustic overstimulation may determine the extent of the hearing loss following loud sound.

Pigmented guinea pigs (200–250 g) were anaesthetized with pentobarbital sodium, Operidine and Droleptan (Janssen) before the animals were prepared for intracellular recording<sup>1,7</sup>. Acoustic test stimuli were delivered in a closed sound system with a Beyer DT48 earphone and the intense tones delivered by a Brüel and Kjaer 12.5-mm condenser driver (Type 4134). The frequency of the intense tone was fixed at 12.5 kHz (110 dB sound pressure level (SPL), 225 ms), which has been shown<sup>3</sup> to be the most effective stimulus for producing a loss of neural sensitivity in the region of the cochlea (15–20 kHz) from which the majority of IHCs were recorded. For the duration of the experiment cochlear sensitivity was assessed by the threshold response of the auditory nerve to gated tones (1 ms rise time). Identification of the hair cells depended on their electrical properties and their recording locations, which were correlated by intracellular dye marking. After a hair cell had been impaled, normal voltage responses were recorded to either a 600-Hz continuous tone (85 dB SPL), or in the case of IHCs, a gated tone (40–60 dB SPL) close to the characteristic frequency (c.f.) of the cell. The animal was then exposed to the 12.5-kHz tone at 1-s intervals and the responses of the hair cell recorded.

IHC receptor potentials (Fig. 1a) generated in response to low-frequency tones can reach 30 mV and demonstrate a depolarizing asymmetry as the intensity increases. As the stimulus frequency increases, the amplitude of the phasic component of the receptor potential decreases at about 6 dB per octave, probably as a direct result of the low-pass electrical properties of the hair cell membrane<sup>1</sup>. For high stimulus frequencies, at or near the c.f. of the cell, the receptor potential produced by IHCs is recorded as a direct current (d.c.) component that can reach 15 mV in amplitude (Fig. 1b). It is thought that this depolarizing potential generated across the hair cell membrane controls transmitter release at the afferent synapse and subsequent afferent fibre excitation<sup>1,7</sup>.

Resting membrane potentials of OHCs in the basal coil of the cochlea are significantly larger than those of IHCs (typically –70 to –85 mV compared with –45 mV) whereas the receptor potentials in response to low-frequency stimuli reach maxima of only 8–10 mV. With low-frequency stimulation the receptor potentials of OHCs are symmetrical at low intensities but, in contrast to IHCs, grow in the hyperpolarizing phase as the stimulus intensity increases (Fig. 1c). For tones close to the presumed c.f. of the cell no d.c. receptor potential is recorded for stimulus levels less than 90 dB SPL (Fig. 1d). A small

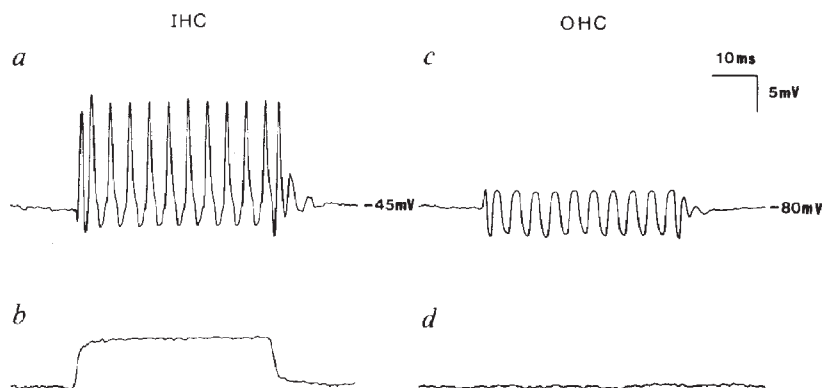
depolarizing d.c. component (up to 5 mV) may be recorded but only when the intensity of the stimulus approaches 110 dB SPL. It thus appears that the transfer characteristics of the IHCs and OHCs in the basal turn are quite different.

At the onset of the 12.5 kHz exposure tone (Fig. 2a), the IHC depolarizes to a level 10–20 mV above the resting potential at a rate that is indistinguishable from the rise of the tone burst (1 ms). The d.c. receptor potential then adapts for the remainder of the exposure. In a number of animals the adaptation demonstrated two separate stages (Fig. 2d), an initial rapid phase with a time constant of about 10 ms and then a slower phase where the cell repolarized at a rate of approximately  $1 \text{ mV s}^{-1}$ . At the offset of the loud tone, the membrane rapidly repolarizes to the pre-exposure resting potential with no obvious sustained elevation of the membrane potential (Fig. 2d). The d.c. voltage responses to the test tone at their best frequencies are reduced after the loud tone and recover between the acoustic overstimulations in a manner that can be described by a single exponential function with a time constant of about 200 ms. With multiple presentations of the loud tone these voltage responses show a cumulative decrease in amplitude. This loss of sensitivity is also seen as a decrease in the amplitude of the compound action potential recorded from the auditory nerve; that is, with successive loud tones the cochlear shows a progressively greater loss of sensitivity.

The growth of the depolarizing d.c. receptor potentials recorded from the OHC (Fig. 2b) during the loud tone differs from the IHC responses in that they demonstrate a considerably longer time constant (4–5 ms) at the onset. In addition, the OHC membrane potential shows no sign of adapting but continues to depolarize during the tone. At the offset the OHC repolarizes, but only to a level above the pre-exposure or control value. With multiple exposures this results in a cumulative depolarization of the cell. For the cell shown in Fig. 2c, this depolarization accumulates at the rate of 0.3 mV after each presentation. However, the membrane potential does return to the control level if sufficient recovery time is allowed between the 12.5-kHz tones.

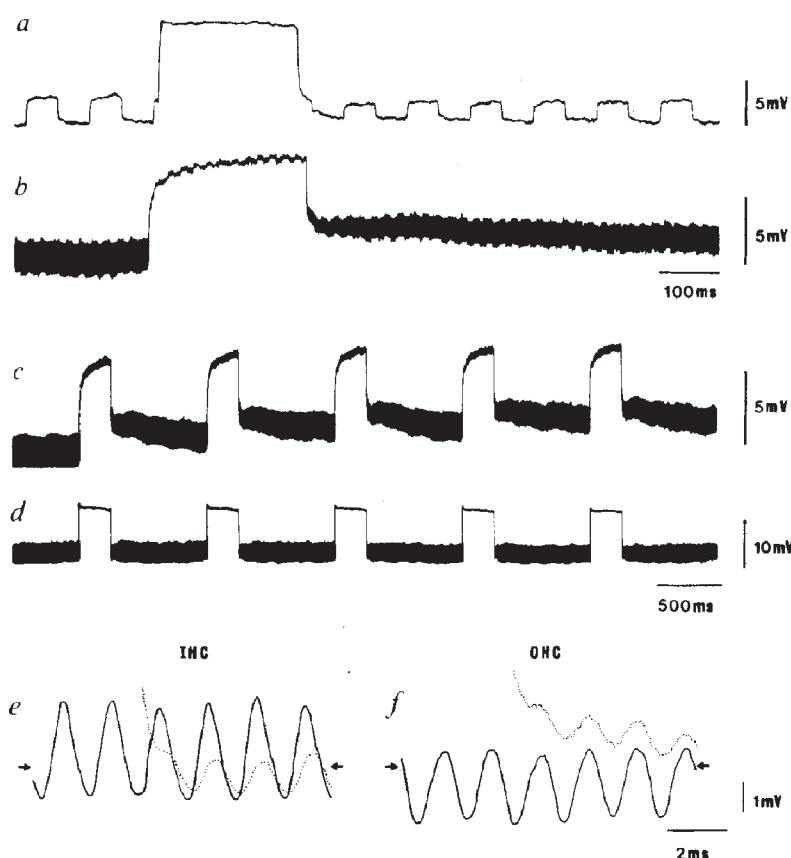
The recovery of the IHC d.c. responses to tones close to their best frequencies is closely correlated ( $r^2 = 0.97$ ) with the repolarization of the OHCs following an intense tone (Fig. 3). OHCs in the basal turn of the cochlea do not generate voltage responses to tones at the best frequencies of the adjacent IHCs, so it was not possible for us to compare the recovery in sensitivity of the OHCs with their membrane potentials at these frequencies. However, we were able to make this comparison at low frequencies where it was possible to monitor the phasic voltage responses of OHCs and IHCs to 600-Hz continuous tones before, during and after exposure to intense tones. The voltage responses of OHCs (Fig. 2b) were reduced following the intense tones and recovered in parallel with the repolarization of the membrane potential. The simplest explanation for this reduction

**Fig. 1** Receptor potentials of an IHC and OHC recorded in the basal coil of the guinea pig cochlea in the same animal with the same electrode. *a*, *b*, Intracellular recordings from IHCs in response to a gated 200-Hz tone (*a*) and 16-kHz tone (*b*) at intensities of 85 dB SPL and 65 dB SPL respectively. In both cases an asymmetrical depolarizing potential is generated during the acoustic stimuli. Recordings from identified OHCs at 85 dB SPL show hyperpolarizing potentials during the 200-Hz tone (*c*) and no depolarizing potential during the 16-kHz (65 dB SPL) (*d*) presentation. OHC responses are in phase with the cochlear microphonic recorded at the round window while the response of IHC phase leads the OHCs by about 90° for a stimulus frequency of 200 Hz. The transients associated with the onset and offset of the hair cell response are derived from transients in the gated tone. All records are single traces and are not averaged.





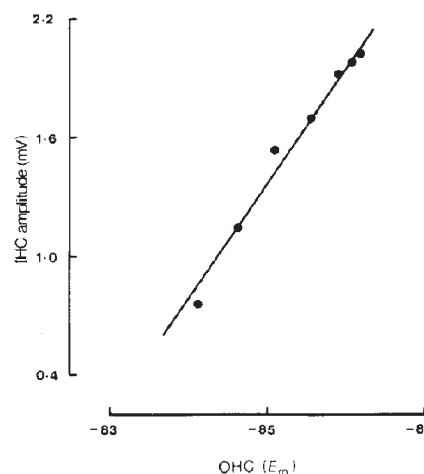
**Fig. 2** *a*, Receptor potentials recorded from an IHC in response to a 16-kHz test tone (60 dB SPL) and a single 225-ms 12.5-kHz exposure. The voltage responses of the IHC to the 16-kHz tones decrease immediately following the loud tone and then recover with a time constant of approximately 200 ms. *b*, OHC voltage responses to a continuous 600-Hz tone (85 dB SPL) and a single presentation of the 12.5-kHz tone. The OHC continues to depolarize during the loud tone and at the offset, the phasic component of the receptor potential is reduced in comparison with the pre-exposure amplitude. It then recovers with a time course similar to that of the IHC. *c*, *d*, Voltage responses recorded in OHC and IHC respectively following five successive exposures to the loud tone. The IHC (*d*) demonstrates no sustained elevation of the membrane potential while the OHC (*c*) is progressively depolarized with each exposure. *e*, IHC receptor potential evoked by a 600-Hz (85 dB SPL) tone before (continuous line) and immediately after acoustic overstimulation (dotted line). The latter demonstrates a decrease in amplitude and an increase in symmetry of the receptor potential around the resting membrane potential (arrow) and a phase lag when compared with pre-exposure recordings. The most significant decrease in amplitude is in the depolarizing phase. This decrease is not associated with a change in the resting membrane potential. *f*, OHC receptor potential recorded before (continuous line) and immediately following (dotted line) the loud tone. The decrease in amplitude and increase in symmetry of the OHC receptor potential is qualitatively similar to that recorded for the IHC but the OHC also demonstrates a prolonged depolarization of the membrane potential that recovers in parallel with the sensitivity of the receptor potential. There is no obvious phase change in the waveform as recorded in the IHC. In contrast to the IHC, the OHC shows a greater loss of amplitude for the hyperpolarizing phase of the receptor potential. All records are single traces with no averaging.



is a reduction in the driving voltage for the transducer current as a result of the OHC depolarization. However, measurements of the membrane impedance of the OHCs after the loud tone showed that the depolarization was not associated with an obvious conductance increase. It is possible that the decrease in the OHC receptor potential amplitude is the result of an increase in the intracellular levels of cations, where cation influx exceeds cation efflux during the intense tone. There is a corresponding, but smaller, reduction in the amplitude of the IHC phasic response after the loud tone (Fig. 2*d*), but in contrast to the OHCs this was not associated with a persistent depolarization of the IHC membrane potential. Furthermore, it was clear that the voltage responses of IHCs were more vulnerable to acoustic overstimulation at their best frequency.

For both groups of cells the reduction in amplitude of their voltage responses to low frequency tones is greatest for one particular phase, depolarizing for the IHC (Fig. 2*e*) and hyperpolarizing for the OHC (Fig. 2*f*), which resulted in the increased symmetry of their waveforms. A phase delay of the IHC receptor potential of about 60° when compared with control responses was also recorded (Fig. 2*e*).

The primary source of mechanical input to cochlear hair cells is the relative shear between the tectorial membrane and their apical surfaces caused by basilar membrane motion during rarefactions (excitatory) and compressions (inhibitory) of the acoustic stimuli<sup>7,8</sup>. This causes displacement of their stereocilia and modulation of their transducer conductances<sup>8-10</sup>. Current theories of cochlear transduction propose that the OHCs, by virtue of the firm attachment of their stereocilia to the tectorial membrane<sup>11</sup>, are in a position to regulate this displacement through a bidirectional mechano-electrical feedback loop<sup>4-6</sup>. That is, OHCs are both detectors and effectors<sup>12,13</sup> and can modify the system that drives them by feedback. In contrast, the IHCs are considered as primarily sensory cells responding to the shear of their free-standing stereocilia<sup>11</sup> through a fluid coupling to basilar membrane motion<sup>7,9</sup>.



**Fig. 3** OHC membrane potential ( $E_m$ ) and the d.c. component of the IHC receptor potential (16 kHz) plotted over a 800-ms recovery period following exposure to a single 225-ms loud tone ( $r^2 = 0.97$ ).

Our most important new finding is that OHCs are depolarized during and following acoustic overstimulation and that their repolarization parallels the recovery of the sensitivity of the IHCs and their afferent innervation. However, if OHCs are the protagonists in a mechano-electrical feedback loop, we do not know if the depolarization is the primary cause of the changes we have observed in the receptor potentials of the IHCs and OHCs during noise-induced hearing loss, or if it reflects more fundamental changes. In light of recent *in vitro* experiments on cochlear hair cells, there are two possibilities worth considering. One is that the primary site of such hearing losses are mechanical structures associated with the OHCs (stereocilia<sup>14,15</sup> and the tectorial membrane<sup>6</sup>), and that the depolarization of the OHCs is a response to the way these mechanical structures function

after overstimulation. The other possibility is that the OHC depolarization is a result of a massive influx of cations during the loud tone, and that one of these ( $\text{Ca}^{2+}$ ) has interacted with contractile and structural proteins in the OHC<sup>16</sup>. This may lead to an alteration in the coupling of the stereocilia to the tectorial membrane, thereby resulting in a decrease in the mechanical input to the IHC. In this case the animal has suffered a noise-induced hearing loss.

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## Human T-cell lymphotropic virus type-I antibodies in Falashas and other ethnic groups in Israel

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Epidemiological studies of the human T-cell leukaemia/lymphoma virus type I (HTLV-I), a type-C retrovirus of the human T-lymphotropic virus family, have used serological surveys to identify population subgroups possessing a high prevalence of naturally occurring HTLV-I-specific antibodies. Studies carried out to delineate the global distribution of the virus have demonstrated natural antibodies to HTLV-I in the serum of healthy donors from specific geographical areas, and have defined viral endemic areas in Japan<sup>1–4</sup>, the Caribbean basin<sup>5–7</sup>, Africa<sup>8–10</sup> and the southeastern United States<sup>11</sup>. Such studies have suggested that the prevalence of HTLV-I antibodies is directly correlated with age<sup>3,12–14</sup>, is associated with the clinical syndrome of adult T-cell lymphoma<sup>1–3,13,15–19</sup>, and is associated with transmission from mother to child<sup>11,12,20</sup>. A separate subtype of the human retrovirus, HTLV-II (refs 21, 22), has also been identified. The population of Israel in part comprises groups of immigrants of various ethnic and geographical origins. Because of this, and the fact that Israel has a highly developed public health system, we surmised that the ethnic groups in Israel could be used in a seroepidemiological survey of HTLV infection. The serological survey reported here demonstrates a high prevalence of HTLV-I antibodies in new immigrants from Ethiopia. This previously ethnically and geographically isolated group, the 'Black Jews' or 'Falashas', from the Gondar region in the northern rural highlands of Ethiopia, has the highest endemic rate of HTLV-I yet reported outside Japan.

Analysis of 1,048 serum samples from Israelis of different ethnic/geographical origins revealed major differences in the

**Table 1** Prevalence of antibodies to HTLV-I in various immigrant groups in Israel

| Ethnic or geographical origin of serum donors             | No. positive/<br>no. tested | %<br>Positive |
|-----------------------------------------------------------|-----------------------------|---------------|
| I, Ethiopia                                               | 86/233                      | 36.9          |
| II, North Africa<br>(Morocco, Tunisia,<br>Algeria, Libya) | 7/168                       | 4.2           |
| III, Middle East: Yemen                                   | 2/30)                       |               |
| Iraq                                                      | 1/61) 4/109                 | 3.7           |
| Iran                                                      | 1/18)                       |               |
| IV, Israeli-born (Ashkenazi)                              | 3/150                       | 2.0           |
| V, Western Europe (Ashkenazi)                             | 3/265                       | 1.1           |
| VI, Eastern Europe (Ashkenazi)                            | 1/83                        | 1.2           |
| Other                                                     | 3/40                        | NS*           |

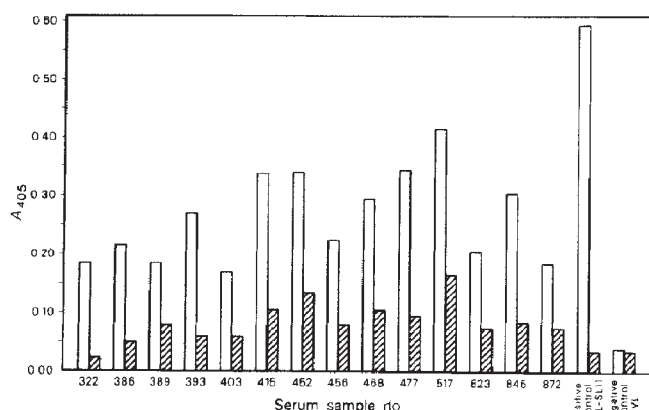
We collected 1,048 serum samples randomly from apparently healthy donors of ages 19–65 yr, male and female, in Magen David Adom (Israeli blood bank) stations, mobile stations and army camps. Blood was drawn for the purpose of routine donation of blood. Sera were obtained from clotted samples remaining after routine clinical phlebotomy. Sera were stored frozen at  $-70^{\circ}\text{C}$  and shipped on dry ice to San Diego for assay. The information collected from the blood donors included age, country or region of birth, origin (ethnic and national) of the donors and their parents, and year of immigration to Israel. For the purpose of this survey, the donors were divided into six groups of ethnic and national origin: I, Ethiopia; II, North Africa (Morocco, Tunisia, Algeria, Libya); III, Middle East (Yemen, Iraq, Iran); IV, Israeli-born (predominantly of Ashkenazi descent); V, Ashkenazis born of Western European descent; VI, Eastern European-born Ashkenazis. All serum samples were collected in the spring of 1984, before the entry of thousands of additional new immigrants from Ethiopia in October–December 1984. Antibodies against HTLV-I antigens were detected by ELISA as developed by Cytotech Inc. HTLV-I was collected from the supernatant fluid of C10/MJ2 cells (provided by Dr R. C. Gallo). Virus-producing cells were pelleted by centrifugation at 3,500 r.p.m. in a RC 3 B centrifuge and the cell-free supernatant was filtered through a 0.45- $\mu\text{m}$  Millipore filter. The extracellular virus was concentrated 50-fold by Pellicon Ultrafiltration (Millipore) and the concentrated virus pelleted at 25,000 r.p.m. on a cushion of 50% Renografin (Squibb). Virus was resuspended in phosphate-buffered saline (PBS) and layered onto a 15–50% Renografin gradient. Centrifugation was for 18 h at 25,000 r.p.m. in a SW28 rotor in a OTD 50 B Sorvall Ultracentrifuge. After gradient fractionation, the virus was resuspended in PBS and pelleted for 1 h at 25,000 r.p.m. Virus purified by this procedure is exceptionally pure and contains <2% non-viral protein (by weight). Solid-phase enzyme immunoassays were used for the detection of HTLV-I antibodies. Each donor serum sample was diluted 1:100 and assayed in duplicate on three separate days. A goat anti-human IgG (heavy + light chains) conjugated to glucose oxidase was used as a second antibody. To each microtitre well was added 200  $\mu\text{l}$  of an enzyme substrate consisting of 200  $\mu\text{l}$  2,2'-azino-bis-3-ethylbenzthiazolinesulphonic acid, 200  $\mu\text{l}$  horseradish peroxidase at 1 mg  $\text{ml}^{-1}$ , 3 ml of 18%  $\beta$ -D-glucose, in 25 ml 0.1 M phosphate buffer pH 6.0, and the development of a green colour was determined at 405 nm after exactly 30 min incubation in a Multiscan (Flow Laboratories) automated spectrophotometer. A reading of twice the background reading obtained with HTLV-negative sera in the same test was considered positive. In an attempt to preclude any false-positive serum antibody determinations, all positive sera were titrated and tested in a competition assay in which the serum samples were preabsorbed with purified HTLV-I virion antigen before being retested in the HTLV-I ELISA. Aliquots (250  $\mu\text{l}$ ) of a 1:100 dilution of donor serum samples were mixed with 1  $\mu\text{g}$  of purified HTLV-I virus in 5  $\mu\text{l}$  and incubated for 60 min at room temperature on a rotary shaker. After absorption with purified virus, the virus-antibody complexes were assayed for unabsorbed antibody by solid-phase immunoassay in duplicate. As a positive control serum in these assays, we used the HTLV-I antibody-positive serum SRL-SL11, a serum from a confirmed case of adult T-cell leukaemia from Japan. Preabsorption of confirmed HTLV-I-positive sera with our purified HTLV-I preparations completely removed all reactivity from these sera.

\* Serum samples from a variety of sources, data not significant.

prevalence of antibodies to HTLV-I (Table 1). Of the 1,008 sera that were divided into ethnic groups I to VI in Table 1, 104 sera were positive for HTLV-I antibodies and 86 of these sera (83%) were donated by immigrants of Jewish Ethiopian (Falasha) descent. Thus, 37% of the sera from Ethiopian donors were positive for HTLV-I antibodies. Groups II and III, consisting of donors originating in North Africa and the Middle East, showed a 10-fold lower prevalence of antibodies to HTLV-I (4.2% and 3.7%, respectively). The remaining groups exhibited levels of prevalence (1–2%) similar to those found in non-endemic areas of Europe, the United States and the higher socioeconomic groups in urban Venezuela<sup>11–12,23</sup>.

Serum samples that were positive for antibodies against HTLV-I in an enzyme-linked immunosorbent assay (ELISA) were further assayed in an absorption test in which the sera were preabsorbed with titrated, saturating concentrations of purified HTLV-I virion antigen, followed by assay of any non-complexed antibody in an HTLV-I ELISA. Whereas the





**Fig. 1** Absorption of HTLV-I-positive Falasha serum samples with purified HTLV-I virions. Open bars, unabsorbed serum samples; cross-hatched bars, HTLV-I-absorbed samples. See text for a discussion of the methods. All serum samples were assayed at a dilution of 1:100.

antibodies that were present in most of the positive serum samples could thus be shown to be specific for HTLV-I, some 30% of the serum samples donated by Falasha individuals could not be absorbed completely with purified HTLV-I antigen. Figure 1 shows the results of absorption experiments with 14 positive Falasha serum samples; a control serum having a high titre of antibodies against HTLV-I (SRL-SL11) and an HTLV-I-negative control serum (VL) were included in the tests. We found that antibodies from 4 of the 14 Falasha serum samples shown (samples 415, 452, 468 and 517) were incompletely absorbed although preabsorption with HTLV-I virions completely absorbed the antibodies from the high-titre control serum. For other serum samples (for example, samples 322, 386, 393 and 403), antibodies to HTLV-I were absorbed efficiently.

It is striking that in this serological survey of HTLV-I infection among immigrant groups in Israel, individuals of one ethnic group should display such a high prevalence of antibodies to HTLV-I and, by inference, have experienced a high frequency of virus infection. Our results show that 37% of the Black Jews of Ethiopian descent, most of them newcomers to Israel of 1 to 2 years at the time of sample collection, had been exposed to HTLV-I and had antibodies to the virus. Healthy Ethiopian-born immigrants to Israel thus have the highest frequency of exposure to HTLV-I encountered or reported to date outside Japan<sup>4</sup>. We are uncertain whether a similar prevalence occurs among non-Jewish Ethiopians in the highlands of that country or in other geographical rural and urban Ethiopian areas. Additional studies in other East African countries may reveal the origin of HTLV and may give rise to the isolation of variant subtypes of HTLV.

In competition immunoassays with purified HTLV-I virions as well as with purified HTLV-II virions (not shown), some HTLV-positive Falasha serum samples could be only partially absorbed. There are two possible explanations for this lack of absorption; either most antibodies in these serum samples are directed against viral core proteins p24, p19 and p15 which are not as readily available in the absorbing whole virions as are the viral envelope antigens, or the unabsorbed sera contain antibodies directed against a new subtype of HTLV which has cross-reacting antigenicities with HTLV-I, but also contains a unique antigenicity. We will investigate these possibilities using alternative methods.

HTLV-I has been shown to be associated with the development of certain T-cell leukaemias and lymphomas<sup>2,5,13,18,19,24</sup>. However, no direct causal relationship has been found between infection with HTLV-I and subsequent development of leukaemia or lymphoma. Unequivocal evidence linking HTLV-I infection with the development of human malignant disease has been hampered by the relatively low incidence of most cancers as well as by the low incidence of infection of most human populations with HTLV. Together with the long incubation

period between exposure to HTLV and the possible development of disease, these difficulties have made prospective studies difficult because of the large number of persons who must be kept under observation. However, the Ethiopian immigrants to Israel, 37% of whom are positive for HTLV, may provide a practical population laboratory for prospective studies linking HTLV infection with subsequent disease. Studies concerning the modes of transmission of HTLV in a human population should also be feasible now that some 12,000 Ethiopian Jewish immigrants have moved to Israel and are living under improved hygienic conditions, with a modern public health system. These directions are being explored.

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## Thymocyte subpopulation enriched for progenitors with an unrearranged T-cell receptor $\beta$ -chain gene

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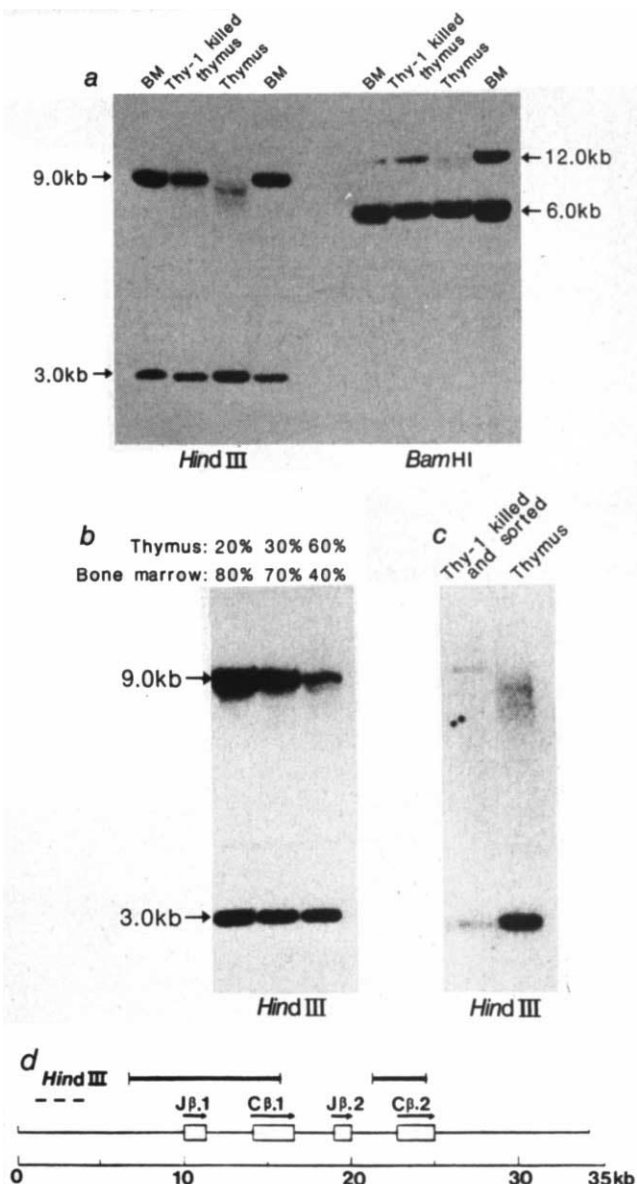
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The development of T cells within the thymus is not well understood. It is known that thymocytes are derived from a progenitor cell in the bone marrow, the prothymocyte<sup>1-5</sup>, and that cells in the subcapsular area of the thymus can give rise to progeny in both the cortex and the medulla<sup>6</sup>. However, it is not clear whether all medullary thymocytes are necessarily derived from cortical cells<sup>7,8</sup>. In particular, it has been difficult to distinguish intrathymic progenitor cells. Recently, however, Lesley *et al.*<sup>9</sup> have defined a thymocyte subpopulation which can be isolated by treatment of the thymus with cytotoxic anti-Thy-1 antibodies and that seems to be enriched for thymocyte progenitors as measured first by its ability to repopulate transiently the thymus of an irradiated host, and second, by its high content of cells bearing Pgp-1 (refs 10, 11), a cell-surface glycoprotein of relative molecular mass 95,000 that is present on most or all prothymocytes of the bone marrow and on fetal thymocytes<sup>29</sup>, but on only a few per cent of cells in the adult thymus<sup>30</sup>. We show here that the gene encoding the  $\beta$ -chain of the T-cell receptor for antigen, which is rearranged during T-cell ontogeny<sup>12-15</sup>, is predominantly in the germline configuration in these cells.

In the initial series of experiments, mouse thymocytes were treated in the presence of complement with a mouse anti-Thy-1.2

**Fig. 1** *a*, Southern blots of Thy-1-killed thymus compared with bone marrow (BM) and total thymus. All lanes contained 10  $\mu$ g digested DNA except the second BM sample which was 20  $\mu$ g. *b*, Reconstruction experiment in which bone marrow DNA and thymus DNA were mixed in the proportions indicated. A total of 10  $\mu$ g DNA was applied to each slot. *c*, Comparison of the Southern blots obtained with total thymus and Thy-1-killed thymus from which RB6-8C5-positive cells had been removed by fluorescence-activated cell sorting. Approximately 2  $\mu$ g DNA from the Thy-1-killed and sorted population and 10  $\mu$ g DNA from total thymus were loaded. *d*, Map of mouse germline T-cell receptor  $C_{\beta}$ -chain genes and their associated  $J_{\beta}$  segments indicating the origin of the *Hind*III restriction fragments observed in *a-c*. The map is derived from data presented in ref. 17.

**Methods.** Thymocytes from 50–80 C57BL/6 mice were treated with the mouse monoclonal anti-Thy-1.2 antibody HO 13.4.9<sup>25</sup> and complement as described in ref. 9. Briefly,  $8-13 \times 10^6$  thymocytes were washed, treated with Tris-buffered ammonium chloride, and samples of  $1 \times 10^9$  cells were spun down. Each cell pellet was resuspended in a total volume of 10 ml of HEPES-buffered Dulbecco's modified Eagle's medium with 2% newborn calf serum and containing tissue culture supernatant of HO 13.4.9 antibody (1:4 final dilution) and rabbit complement (1:20 final dilution). After incubation at 37°C for 45 min, the cells were overlaid on 15 ml of Ficoll-Hypaque (Pharmacia) and centrifuged at room temperature for 25 min at 1,700g. The interface layer was recovered and washed. In some experiments, in which significant numbers of dead cells remained, the Ficoll-Hypaque treatment was repeated. After killing and Ficoll-Hypaque treatment, 0.2–0.4% of the input cells were recovered. A portion of the recovered cells was taken for flow cytometry (see Fig. 2 legend). High relative molecular mass DNA was prepared from the remaining cells by standard procedures and digested completely with either *Bam*HI or *Hind*III<sup>26</sup>. The digested DNA was fractionated on a 0.7% agarose gel and transferred to nitrocellulose filters. Filters were hybridized at 42°C overnight with the <sup>32</sup>P-labelled *Eco*RI fragment of the 86T5 murine  $\beta$ -chain plasmid (0.25  $\mu$ g;  $2 \times 10^8$  c.p.m. per  $\mu$ g) in 40% v/v formamide, 4  $\times$  S.S.C., 10% w/v dextran sulphate, 0.8  $\times$  Denhart's solution, 500  $\mu$ g ml<sup>-1</sup> salmon sperm DNA in 7 mM Tris-HCl pH 7.6. Filters were washed twice for 30 min at room temperature, with 2  $\times$  SSC/0.1% SDS and then washes were repeated with 0.1 SSC/0.1% SDS at 55°C. Autoradiography was for 2–5 days.

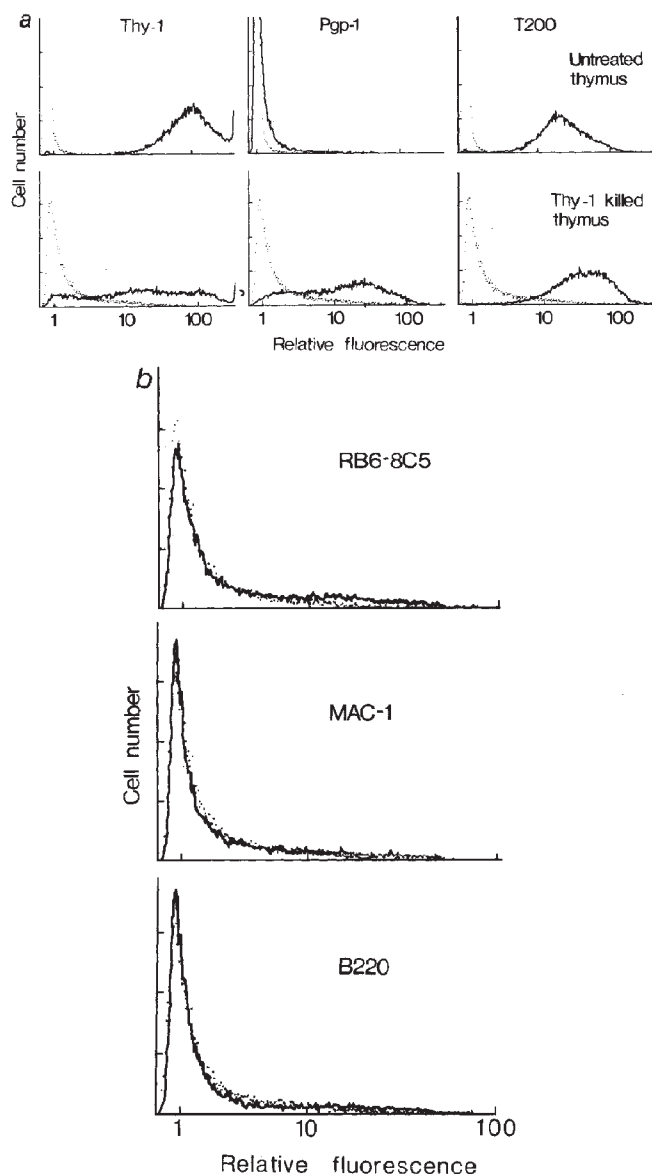


monoclonal antibody (see Fig. 1 legend). After incubation, dead cells were removed by centrifugation over Ficoll-Hypaque and a portion of the viable cells was used to prepare high relative molecular mass DNA. The remaining cells were then stained with rat monoclonal antibodies against cell-surface antigens and analysed by flow cytometry using as a second-stage reagent a fluoresceinated goat anti-rat IgG which does not crossreact with mouse IgG. This made it possible to determine the cell-surface phenotype of the cell population in the presence of the mouse anti-Thy-1 antibody used for cytotoxic ablation. Concomitantly, by hybridizing DNA from these cells by the method of Southern<sup>16</sup> with a <sup>32</sup>P-labelled mouse T-cell receptor  $\beta$ -chain probe, it was possible to determine whether the T-cell receptor  $\beta$ -chain gene had undergone rearrangement in these cells. Figure 1a shows a typical experiment in which DNA from bone marrow, unfractionated thymus, and thymocytes surviving treatment with the cytotoxic anti-Thy-1 antibody had been digested with either *Hind*III or *Bam*HI restriction enzymes. In both cases, two bands hybridized to the  $\beta$ -chain probe, 86T5, which is an *Eco*RI cDNA fragment spanning the V-J (variable-joining) and C (constant) regions of the gene<sup>14</sup>. Comparison of the pattern obtained with total thymus and bone marrow shows the clear-cut distinction between the non-rearranged and rearranged genes. Whereas the higher relative molecular mass band obtained with both restriction enzymes is sharp when bone marrow DNA is probed, the total thymus gives a heterogeneous

smear of bands. Most strikingly, the autoradiograph also clearly shows that the T-cell receptor  $\beta$ -chain gene in the 0.5% of thymocytes surviving treatment with anti-Thy-1 antibody and complement is predominantly non-rearranged. From reconstruction experiments using bone marrow and total thymus DNA in different proportions (Fig. 1b), it can be estimated that >80% of the T-cell  $\beta$ -chain genes in this thymocyte subpopulation are non-rearranged. The pattern of bands obtained from the *Hind*III digests are particularly informative. Previous work has shown that the 9-kilobase (kb) fragment extends upstream of the  $J_{\beta}$ 1 segment to the 3'-end of the  $C_{\beta}$ 1 gene (Fig. 1d and refs 17–19). Thus, this restriction fragment is variably reduced in size during diversity-joining rearrangements in the T-cell receptor  $\beta$ 1-chain cluster. In contrast, the 3-kb fragment is derived from the C region of the  $\beta$ 2-chain gene and is unaffected by rearrangements within the  $\beta$ 2-chain gene cluster. Consequently, deletions in the 9-kb fragment serve as an indicator of all rearranged  $\beta$ 1-chain genes, whereas the 3-kb fragment derived from the  $C_{\beta}$ 2 region serves as an internal control.

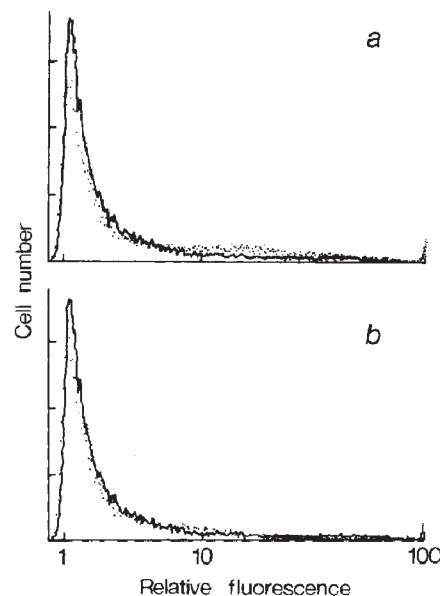
The antigenic phenotype of the thymus subpopulation was determined with two objectives in mind: first, to determine whether the phenotype of these cells differed from that of the bulk population of thymocytes, and second, to estimate the extent to which the cells surviving treatment with anti-Thy-1 antibody may be derived either from non-lymphoid thymic tissue or from mature haematopoietic cells derived from either blood





**Fig. 2** Surface antigens of the anti-Thy-1-killed thymocyte population analysed by flow cytometry. Specific fluorescence (solid curves) is expressed relative to the peak of the background population in which normal tissue culture medium was substituted for monoclonal antibody containing tissue culture supernatant (dotted curves). *a*, Relative to untreated thymus, Thy-1-killed thymocyte populations show heterogeneous staining for Thy-1 with a subpopulation at or near background levels. Thy-1-killed thymocytes are enriched in highly fluorescent Pgp-1<sup>+</sup> cells and are essentially all T200<sup>+</sup>. *b*, Most cells in Thy-1-killed populations do not stain with antibodies recognizing mature granulocytes (RB6-8C5), macrophages and monocytes (Mac-1), or B cells (B220). Ordinate: cell number.

**Methods.** Aliquots of  $1 \times 10^6$  untreated thymocytes and  $2-5 \times 10^5$  thymocytes recovered after cytotoxic treatment with the mouse monoclonal anti-Thy 1.2 antibody, HO 13.4.9, and centrifugation on Ficoll-Hypaque as described in Fig. 1 legend ('Thy-1-killed thymocytes') were stained with tissue culture supernatants of the rat anti-mouse hybridomas C22/22.7.1.1 (ref. 27) (anti-Thy-1), IM7.8.1 (ref. 10) (anti-Pgp-1), I3/2.3<sup>20</sup> (anti-T200), RB6-8C5, which recognizes mature granulocytes (R. Coffman *et al.*, in preparation), M1/70.15<sup>22</sup> (anti-Mac-1), and RA3-3A1<sup>21</sup> (anti-B220), followed by staining with a fluorescein-labelled goat anti-rat immunoglobulin which had been absorbed to remove antibodies reacting with mouse immunoglobulin (Hyclone). Flow cytometry was carried out on the Salk Institute flow microfluorimeter. Data were stored as list mode data files which were gated to remove dead cells stained with propidium iodide as detected in the red channel. Fluorescein fluorescence in the green channel was log-compressed with a three-decade logarithmic amplifier before analog to digital conversion as previously described<sup>28</sup>.



**Fig. 3** Thy-1 population sorted for RB6-8C5-negative cells. *a*, Sorted (solid curve) and not sorted (dotted curve) Thy-1-killed thymocytes stained for RB6-8C5. The sorted population is depleted in cells staining with the RB6-8C5 antibody. *b*, Sorted cells stained with RB6-8C5 (solid curve) compared with the background staining of the Thy-1-killed population which was not sorted (dotted curve). Specific staining approaches background levels.

**Methods.** Thymocytes were treated as described in the legend to Fig. 1. Thy-1-killed cells ( $1.4 \times 10^7$ ) from the same experiment as that illustrated in Fig. 2 were stained with RB6-8C5 antibody and fluorescent goat anti-mouse immunoglobulin. These stained cells were sorted on the Salk Institute BCS-176 cell sorter with the instrument optimized for the rapid selection of RB6-8C5-negative cells using anti-coincidence logic in the green fluorescence channel. The cells were sorted at a rate of 2,500 cells  $s^{-1}$ , deflecting three drops per event. The cells recovered from the sort were restained with various monoclonal antibodies and fluorescent goat anti-rat immunoglobulin as described in Fig. 2 legend.

or parathymic lymph nodes that may be present in the thymocyte preparations. The antigenic profile which has been consistently seen in four such replicate experiments is shown in Fig. 2. The data show that essentially all the cells express T200 glycoprotein<sup>20</sup> on their cell surface, suggesting that there are few, if any, non-lymphoid cells in the subpopulation of cells surviving treatment with anti-Thy-1 antibody. Moreover, the surviving cell population is enriched for Pgp-1<sup>+</sup> cells, consistent with the hypothesis that Pgp-1 glycoprotein is expressed on thymocyte progenitor cells. Most cells express Thy-1 but at levels much lower than the bulk thymocyte population and further analysis of these data indicates that a small subpopulation of cells expresses little or no Thy-1. With regard to the possible contribution of cells from either blood or parathymic lymph nodes, in this particular experiment ~5% of cells stained with a monoclonal antibody against B220, which is thought to be specific for B cells<sup>21</sup>; 7% stained with a monoclonal antibody against Mac-1, which is considered specific for myeloid cells<sup>22</sup>; and 13% stained with monoclonal antibody RB6-8C5, which is known to stain mature granulocytes (R. Coffman, J. Carty, L. Gemmell, J. Lesley, I. Weissman and D. Rennick, in preparation). On the basis of these results, it can be estimated that in this experiment the maximum contribution of non-T cells in the subpopulation of the thymus surviving treatment with anti-Thy-1 antibody is 25%. This is a maximum estimate since it is not known whether the RB6-8C5 monoclonal antibody reacts with thymocyte progenitor cells. It should also be noted that any contaminating mature T cells from blood or lymph nodes would yield DNA in which the T-cell receptor genes are rearranged.

These results make it unlikely that most of the non-rearranged T-cell receptor  $\beta$ -chain genes found in the subpopulation of

cells surviving treatment with anti-Thy-1 antibody were derived from cells other than thymocytes. However, to address this question more rigorously, an additional set of experiments were carried out. Thymocytes were first treated with anti-Thy-1 antibody and complement and then the viable cells remaining were isolated and stained with monoclonal antibody RB6-8C5. The cells were then fractionated by fluorescent cell sorting to remove cells staining with monoclonal antibody RB6-8C5. As shown in Fig. 3, this procedure reduced the number of cells staining with this antibody approximately 10-fold. The Thy-1 and Pgp-1 profiles of the sorted and nonsorted populations were essentially identical. When DNA from the sorted cells was digested with *Hind*III and blotted, it was clear that most cells had unrearranged  $\beta$ -chain genes although the limited amount of DNA available for analysis precluded precise quantitation (Fig. 1c).

In conclusion, we have shown that the subpopulation of thymocytes surviving treatment with anti-Thy-1 antibody and complement consists largely of cells in which the gene for the  $\beta$ 1-chain of the T-cell receptor has not yet undergone rearrangement. This direct evidence that in the mouse the rearrangement of the  $\beta$ -chain gene is an intrathymic event is consistent with previous data from studies in man<sup>23,24</sup>. The cell-surface phenotype of these surviving cells indicates that the great majority of them are bona fide thymocytes that express Pgp-1 glycoprotein. We believe that the previous data showing that thymocyte subpopulations enriched in Pgp-1<sup>+</sup> cells can transiently repopulate the thymus and that Pgp-1<sup>+</sup> cells are found in abundance in early fetal thymus, together with the observations in the present paper, indicate that, although this population is still heterogeneous, thymocyte progenitor cells are enriched in this subpopulation of thymocytes. It is interesting that recently a thymic subpopulation representing 2–4% of unfractionated cells has been isolated in which the  $\beta$ -chain genes have undergone rearrangement but the  $\alpha$ -chain genes have not (M. Davis *et al.*, personal communication). Although other explanations are possible, these cells may represent the next stage of development of the progenitor cells identified in the present studies.

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## Similarities between interleukin-2 receptor number and affinity on activated B and T lymphocytes

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Interleukin-2 (IL-2) is a T-cell-derived polypeptide hormone of 133 amino acids which exerts its growth-promoting activity via a surface receptor<sup>1</sup>. Originally, IL-2 was believed to be a unique growth factor for activated T cells<sup>2</sup>; more recent studies, however, have demonstrated that certain B-cell tumours<sup>3</sup> as well as normal activated B lymphocytes<sup>4–6</sup> express a surface molecule which is recognized by monoclonal antibodies directed against the IL-2 receptor. Furthermore, we<sup>7</sup> and others<sup>6</sup> have shown recently that activated B cells proliferate in response to either immunoaffinity-purified<sup>8</sup> or recombinant<sup>9</sup> IL-2. These controversial findings prompted us to undertake a detailed quantitative comparison of IL-2 receptor expression on activated B and T cells. We show here, using biosynthetically labelled IL-2 (<sup>3</sup>H-IL-2) and anti-IL-2 receptor antibody (<sup>3</sup>H-PC61) that activated B and T cells express both high-affinity (apparent dissociation constant,  $K_d \sim 20$  pM) and low-affinity ( $K_d \sim 1,000$  pM) IL-2 receptors. Binding of IL-2 to both classes of receptor is inhibited by the monoclonal anti-IL-2 receptor antibody PC61. B blasts express half as many total IL-2 binding sites or PC61 binding sites as T blasts, and the ratio of the number of low- to high-affinity receptors for each cell type is  $\sim 10:1$ . Immunoprecipitation analysis of surface-labelled blasts indicates that B and T cells have IL-2 receptors of similar relative molecular mass. Taken together, these data suggest strongly that IL-2 can act as a growth hormone for both B and T lymphocytes.

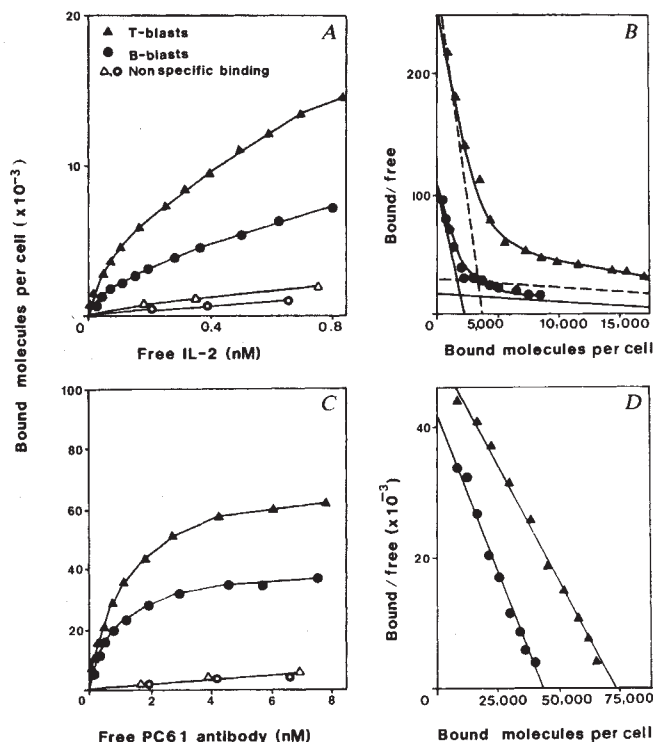
To quantitate and compare the number of IL-2 receptors expressed on activated B and T cells, we used <sup>3</sup>H-IL-2 in equilibrium binding assays<sup>2</sup>. Previous studies have shown that murine B cells activated with lipopolysaccharide (LPS) alone do not respond to<sup>7</sup>, nor do they express detectable receptors for, IL-2<sup>2,7</sup>; however, B cells activated with LPS and anti-immunoglobulin antibodies (anti-Ig)<sup>7</sup> bind <sup>3</sup>H-IL-2 in a specific manner (Fig. 1A). Scatchard plot analysis of the specific binding data (using the Scatfit computer program) demonstrates that the activated B cells, like T cells, express two classes of IL-2 receptor. Figure 1B shows that the B blasts express 2,000 high-affinity IL-2 receptors ( $K_d = 22$  pM) and  $\sim 10$  times as many receptors of relatively lower affinity ( $K_d = 1,330$  pM). Although the numbers of both classes of receptors expressed by concanavalin A (Con A)-activated T cells are about twice those on LPS/anti-Ig-activated B cells, the  $K_d$  values are very similar (17 and 1,270 pM for high- and low-affinity receptors, respectively). Binding of <sup>3</sup>H-IL-2 to low-affinity receptors is saturable at an IL-2 concentration of 3–5 nM (not shown); furthermore, it can be inhibited either with unlabelled IL-2 or with monoclonal antibody PC61 (ref. 10; Fig. 1A) but not with monoclonal antibodies to other cell-surface antigens (not shown). Although previous studies<sup>2</sup> reported that activated T cells express a single class of high-affinity receptors ( $K_d = 5–20$  pM), a more recent report by Robb *et al.*<sup>11</sup> demonstrated that a second class of receptors of lower affinity is also detectable on murine and human T cells when much higher concentrations of IL-2 than those previously tested are used. Our results with various cell types (Table 1) including human phytohaemagglutinin (PHA)-induced blasts (not shown) show a 50–100-fold difference in  $K_d$  between the two classes of IL-2 receptor and an 8–14-fold excess of low-over high-affinity receptors, which is in close quantitative agreement with studies involving other hormone systems such as epidermal growth factor<sup>12,13</sup> and insulin<sup>14,15</sup>. Furthermore, both

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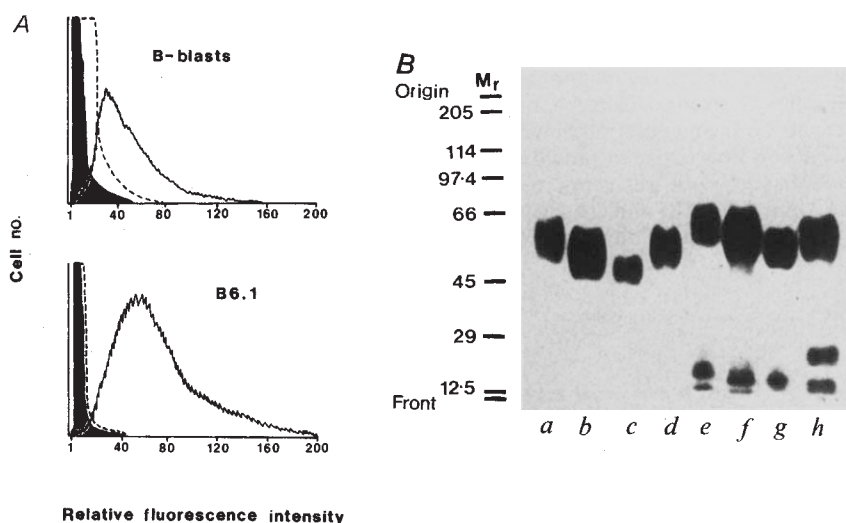


**Fig. 1** Comparison between equilibrium binding of IL-2 and PC61 on B (●) and T (▲) blasts. In the presence of either excess unlabelled IL-2 or PC61 antibody, the binding of  $^3\text{H}$ -IL-2 was reduced by 90–95% at all IL-2 concentrations tested (A, open symbols). The binding of  $^3\text{H}$ -PC61 was inhibited by excess unlabelled PC61 (C, open symbols) but not by unlabelled IL-2 (not shown). Specific equilibrium binding (solid symbols) of IL-2 and PC61 is shown in A and C, respectively, after subtraction of nonspecific binding (open symbols). Scatchard plot analyses of these data were performed using a weighted least-squares curve-fitting computer program<sup>22</sup> (B and D). For IL-2 binding (B), the computer chose a two-binding-site model over a one-site model as the best fit to the experimental data. Broken lines (T blasts) and solid lines (B blasts) represent the computer-generated high- and low-affinity binding components. Similar results were obtained in three independent experiments.

**Methods.** B blasts were prepared as described elsewhere<sup>7</sup> by culturing purified C57BL/6 splenic B cells in the presence of LPS, anti-Ig beads and 10% EL4 supernatant (SN). T blasts were prepared by culturing spleen cells from the same mouse strain at a concentration of  $2 \times 10^6 \text{ ml}^{-1}$  in the presence of  $1.5 \mu\text{g ml}^{-1}$  Con A and 10% EL4 SN. Both cell types were collected after 4 days, centrifuged on a Ficoll/Hypaque gradient and washed extensively. The preparation of affinity-purified  $^3\text{H}$ -IL-2 and the IL-2 binding assay have been described in detail<sup>2,8</sup>. In the experiments described here, all incubations were carried out at  $4^\circ\text{C}$  in the presence of azide. For PC61 binding, aliquots of  $10^6$  cells were incubated for 20 min (equilibrium binding occurs within 15 min) in the presence of various amounts of protein A-Sepharose-purified  $^3\text{H}$ -PC61 (ref. 10) in a volume of 200  $\mu\text{l}$ . Before the antibody binding assay, cells were pre-incubated in the presence of 10% normal rat serum in order to block nonspecific binding of PC61 via Fc receptors. Free and cell-bound PC61 were measured by liquid scintillation counting after centrifugation through a paraffin-silicon oil gradient<sup>7</sup>. The number of bound IL-2 and PC61 molecules per cell was calculated given the specific activities of 2,500 c.p.m.  $\text{ng}^{-1}$  and 210 c.p.m.  $\text{ng}^{-1}$ , respectively. The  $M_r$  of IL-2 and PC61, as judged by SDS-PAGE, is 15K and 170K, respectively.



**Fig. 2** A, Most activated B and T cells express the PC61 antigen. Solid lines represent the fluorescence profile of the PC61 antigen distribution (B blasts on gain 16; B6.1, gain 4); broken lines show the level of nonspecific staining after incubation in the presence of excess unlabelled PC61; shaded areas show background staining with avidin-fluorescein isothiocyanate (FITC) alone. In B-blast preparations, Thy-1-positive cells were not detected above background fluorescence. In addition, we have shown previously<sup>7</sup> by immunoperoxidase staining that cultures of B blasts contain >97% immunoglobulin-positive cells and <0.3% Thy-1-positive cells. The mean fluorescence intensity of B6.1 cells was about five times higher than for B blasts when normalized to the same gain, which is consistent with the data shown in Table 1. B, PC61 immunoprecipitates membrane components of similar  $M_r$  from different types of IL-2-responsive cells. Electrophoretic patterns (lanes a–d, non-reduced; e–h, reduced conditions) were obtained after immunoprecipitation with PC61 of lysates obtained from various types of IL-2-dependent cells: B1.8 (a, e), Con A blasts (b, f), B blasts (c, g) and BD2.10 (d, h). Lane g was exposed for 7 days, and all other lanes for 4 days.



**Methods.** A, B blasts were prepared as described in Fig. 1 legend, centrifuged on a Ficoll/Hypaque gradient, washed and pre-incubated in 10% normal rat serum.  $5 \times 10^5$  B blasts or B6.1 cells were then incubated (20 min at  $4^\circ\text{C}$ ) in 100  $\mu\text{l}$  of phosphate-buffered saline (PBS) containing 2  $\mu\text{g}$  of biotinylated PC61 antibody in the presence or absence of 100  $\mu\text{g}$  unlabelled PC61. The cells were washed, incubated for 20 min with avidin-FITC and analysed on a fluorescence-activated cell sorter (FACS II; Becton and Dickinson) gated to exclude non-viable cells<sup>23</sup>. B, Cells ( $1-2 \times 10^7$ ) were surface-labelled with  $^{125}\text{I}$  by the glucose oxidase-lactoperoxidase method of Hubbard and Cohn<sup>24</sup> as modified by Luescher *et al.*<sup>25</sup>. The labelled cells were lysed in 0.5 ml of cold 1% recondensed Triton X-114 in 50 mM Tris-HCl (pH 7.7) containing 0.3 M NaCl and 5 mM EDTA. The aqueous and detergent phases were separated according to Bordier<sup>26</sup>. Triton-insoluble material was removed by centrifugation of the lysates for 3 min at  $4^\circ\text{C}$ . The lysates were heated to  $30^\circ\text{C}$  for 1 min, then spun in an Eppendorf centrifuge at room temperature for 1 min. The aqueous phase was removed, the detergent phase re-dissolved in 0.5 ml of cold buffer and the separation repeated. The lysates were pre-cleared for immunoprecipitation by sequential addition of 5  $\mu\text{l}$  normal rat serum, 20  $\mu\text{l}$  rabbit anti-rat immunoglobulin and 12.5  $\mu\text{l}$  protein A-Sepharose 4B (twice), each step followed by incubation for 20 min on a rotating mixer. The pre-cleared lysates were then incubated for 60 min with purified PC61 antibody coupled to Sepharose 4B, followed by extensive washing of the lysates with two buffers: (1) 10 mM phosphate buffer (pH 8.2) containing 0.05% SDS, 0.05% deoxycholate, 0.5% Nonidet P-40 (NP40) and 10 mM EDTA; and (2) 0.12 M Tris-HCl (pH 8.2) containing 0.5 M NaCl, 0.5% NP40 and 10 mM EDTA. The immunoprecipitates were analysed by SDS-PAGE according to Laemmli<sup>27</sup> on 7.5–15% gradient gels. Samples were reduced with 0.1 M dithiothreitol and denatured by boiling in 4% SDS. After electrophoresis, the gels were autoradiographed at  $-70^\circ\text{C}$  on Kodak XAR film.  $M_r$  standards were: myosin (205K),  $\beta$ -galactosidase (114K), phosphorylase B (97.4K), bovine serum albumin (66K), ovalbumin (45K), carbonic anhydrase (29K) and cytochrome c (12.5K).

**Table 1** Comparison of the number of binding sites for PC61 antibody and IL-2 on activated B and T cells

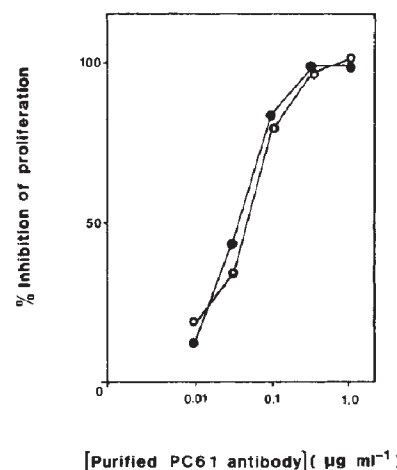
| Cell type<br>(no. of<br>experiments) | IL-2 binding sites            |                   |            | PC61<br>binding<br>sites<br>( $\times 10^{-3}$ ) | Ratio<br>PC61/IL-2<br>binding<br>sites |
|--------------------------------------|-------------------------------|-------------------|------------|--------------------------------------------------|----------------------------------------|
|                                      | Total<br>( $\times 10^{-3}$ ) | Ratio<br>low/high | $K_d$ (pM) |                                                  |                                        |
| B blasts (3)                         | $18 \pm 3$                    | 10                | 23/1,150   | 40                                               | 2.2                                    |
| T blasts (3)                         | $37 \pm 5$                    | 12                | 19/1,360   | 73                                               | 2.0                                    |
| B1.8 (2)                             | $29 \pm 4$                    | 8                 | 17/1,420   | 48                                               | 1.7                                    |
| B6.1 (2)                             | $79 \pm 9$                    | 9                 | 16/690     | 165                                              | 2.1                                    |
| BD210 (3)                            | $166 \pm 14$                  | 14                | 25/1,800   | 285                                              | 1.7                                    |

Equilibrium binding of IL-2 and PC61 antibody was measured as described in Fig. 1 legend and the data processed by computer analysis. For each cell type we show the total number of IL-2 binding sites per cell, the ratio of the number of low- to high-affinity IL-2 binding sites per cell and the  $K_d$  of both classes of receptor (high/low affinity). The number of PC61 binding sites was also calculated by Scatchard analysis. The final column shows the ratio of the number of PC61 antibody binding sites to the total number of IL-2 binding sites per cell. The following IL-2-independent cell lines were found to be negative for specific binding of both IL-2 and PC61; Jurkat, EL4, BW5174, LSTRA, and mouse spleen and thymus cells. B and T blasts were prepared as described in Fig. 1 legend. B1.8 (ref. 20) is an antigen-dependent cytotoxic T lymphocyte (CTL) clone derived from a (Cremona  $\times$  CD) $F_1$  mouse. B6.1 and BD2.10 are two antigen-independent CTL clones derived from C57BL/6 and (C57BL/6  $\times$  DBA) $F_1$  mice, respectively<sup>21</sup>.

the  $K_d$  values that we find for the high-affinity IL-2 receptors and the absolute number of receptors of both classes (Table 1) agree closely with those of Robb *et al.*<sup>11</sup>; however, they report a 10–20-fold lower  $K_d$  (20–40 nM) for the low-affinity IL-2 receptors. Because we have found that the dissociation rate of IL-2 from the low-affinity receptors is very fast ( $t_{1/2} \sim 45$  s at 4°C in medium; our unpublished data), we believe that this discrepancy in the estimates of the  $K_d$  of the low-affinity receptors results from the additional washing steps in the protocol used in the previous study. Regardless of these technical considerations, it still remains to be established whether the low-affinity receptors are biologically functional or whether they are a recycled or degraded form of the high-affinity receptor.

Recently, we have characterized a rat monoclonal antibody, PC61 (ref. 10), which specifically recognizes the murine IL-2 receptor as shown by the fact that it (1) inhibits by 95% the IL-2-dependent proliferation of cloned cytolytic T cells; (2) binds specifically to IL-2-responsive cells; (3) inhibits by >90% (at either 4°C or 37°C) the binding of  $^3H$ -IL-2 to both high- and low-affinity receptors; and (4) immunoprecipitates from surface-labelled activated T cells a band of similar relative molecular mass ( $M_r$ ) to those reported for other anti-IL-2 receptor antibodies<sup>4,5,16</sup>. Figure 1c compares the equilibrium binding of  $^3H$ -PC61 by LPS/anti-Ig-activated B blasts and Con A-activated T blasts; the binding is saturable and can be competed for by excess unlabelled PC61, and Scatchard analysis (Fig. 1d) reveals that both types of cell bind PC61 with equal affinity ( $K_d \sim 1$  nM). At saturation, T blasts bind 73,000 molecules per cell, almost twice the number bound by B blasts, which is consistent with the IL-2 binding data. Our results are also in good agreement with the recent finding that rat and human T blasts bind, respectively, 75,000 and 100,000 anti-IL-2 receptor antibody molecules per cell<sup>17,18</sup>.

To date, it has been difficult to reconcile estimates of the number of IL-2 receptors expressed per cell as measured by IL-2 binding, with those derived from binding of anti-receptor antibody. Table 1 compares quantitative IL-2 and PC61 binding to a variety of IL-2-dependent cells derived from different mouse strains. Even over an almost 10-fold range in total number of receptors per cell, the number of PC61 binding sites is about twice (1.7–2.2) the number of IL-2 binding sites. A similar finding for human cells has been published recently<sup>11</sup>; PHA-blasts and HUT-102 cells were found to have 2.0–2.6 times as many anti-



**Fig. 3** PC61 antibody inhibits the IL-2-dependent proliferation of activated B and T cells. B blasts were prepared as described in Fig. 1 legend, collected on day 3, centrifuged on a Ficoll/Hypaque gradient and washed.  $10^4$  cells were cultured in 200  $\mu$ l of RPMI-1640 medium containing 10% fetal calf serum (FCS). Cloned murine IL-2-dependent T cells (CTLL<sup>28</sup>) were washed three times and seeded at  $4 \times 10^3$  cells per 200  $\mu$ l of Dulbecco's modified Eagle's medium (DMEM) containing 5% FCS. To each well was added a suboptimal amount of IL-2 (giving 75% of maximum response in each assay) and various amounts of purified PC61 antibody. Cell proliferation was measured at 72 h (B blasts,  $\circ$ ) or 24 h (CTLL,  $\bullet$ ) after a 6-h pulse with 1  $\mu$ Ci of  $^3H$ -thymidine per well. A typical experiment is shown here, where symbols represent mean values of triplicate wells (s.e.m. = 10% of mean). Maximum c.p.m. in the absence of PC61 was  $3.3 \times 10^4$  and  $1.6 \times 10^4$  for B blasts and CTLLs, respectively. Thymidine incorporation, in the absence of added IL-2, was 500 c.p.m. for both populations. Similar results were obtained from 48-h cultures of CTLLs. From several experiments, the concentration of antibody giving 50% inhibition of proliferation was in the range 25–50 ng ml<sup>-1</sup> for both cell types.

Tac<sup>18</sup> antibody binding sites as IL-2 binding sites. The reason for this difference is not clear, but a possible explanation is that two antibodies bind to a single IL-2 receptor. Alternatively, the difference could be explained by the binding of PC61 to molecules which do not bind IL-2 with a detectable affinity.

Flow microfluorometric analysis (Fig. 2A) shows that the distribution of PC61 antigen on LPS/anti-Ig-activated B blasts is similar to that seen on an IL-2-dependent cytolytic T-cell clone. Over 95% of the cells express the PC61 antigen. Analysis by SDS-polyacrylamide gel electrophoresis (PAGE) (Fig. 2B) reveals that the PC61 antigen immunoprecipitated from LPS/anti-Ig-activated B cells, T blasts or cloned T cells migrates as a broad band with an apparent  $M_r$  of 50,000–70,000 (50–70K) (reduced form) and 45–65K (non-reduced). This major band is similar to those reported by others using independently derived anti-IL-2 receptor antibodies<sup>4,5,18,19</sup>. In addition, three minor bands of lower  $M_r$  are consistently observed under reducing conditions; one at  $\sim 25$ K and two at 14–17K. The presence of low- $M_r$  bands (20–25 K) has been reported previously for murine T cells<sup>16</sup>. The broad, high- $M_r$  band precipitated from B blasts migrates slightly faster (reduced and non-reduced) than that of T cells and the low- $M_r$  bands are less pronounced (Fig. 2B, lane g). We have also observed bands of similar  $M_r$  following precipitation with the 7D4 anti-IL-2 receptor antibody<sup>4</sup> (provided by Dr T. R. Malek). The heterogeneity in immunoprecipitation patterns observed between the different cell types studied (also reported for a variety of human T cells<sup>19</sup>), necessitates further biochemical analysis, such as peptide mapping, before the fine structure of the IL-2 receptors expressed by activated B and T cells can be compared.

Figure 3 shows that PC61 inhibits the IL-2-dependent proliferation of activated B and T cells at antibody concentrations very similar to those which inhibit the binding of IL-2 to its receptor (unpublished results).



Thus, by several independent criteria, the IL-2 receptors expressed by LPS/anti-Ig-activated B cells and activated T cells are similar. Quantitative  $^3\text{H}$ -IL-2 binding reveals that B blasts, like a variety of independently derived activated T cells, express two classes of IL-2 binding sites; one has a high affinity for IL-2 and the other a 50–100-fold lower affinity. Binding to both classes can be inhibited by the same monoclonal antibody. The ratio of the number of low- to high-affinity sites per cell for B blasts (10) is within the range found for activated T cells (8–14). In addition, studies using antibody PC61 show that: (1) >95% of activated B and T cells express IL-2 receptors; (2) IL-2-dependent proliferation of both cell types is inhibited at similar anti-

body concentrations; and (3) bands of similar  $M_r$  are immunoprecipitated from activated T and B cells. On the basis of these results, we propose that IL-2 promotes the growth of appropriately stimulated B and T cells via a common mechanism.

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## $\gamma$ -Interferon transcriptionally regulates an early-response gene containing homology to platelet proteins

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Interferons are a family of proteins first identified by their ability to induce cellular resistance to infection by many viruses. In addition to the antiviral properties it shares with the  $\alpha$ - and  $\beta$ -interferons,  $\gamma$ -interferon (IFN- $\gamma$ ), a lymphokine secreted by activated T cells, activates macrophages, stimulates B cells, increases fibroblast and endothelial cell resistance to many non-viral intracellular parasites and modulates cell-surface proteins central to immune cell regulation<sup>1–13</sup>. To identify molecules involved in the IFN- $\gamma$  response and characterize their modulation, we have isolated genes that are induced following recombinant IFN- $\gamma$  treatment of U937 cells, a histiocytic lymphoma cell line with monocytic characteristics<sup>14,15</sup>. We report here the molecular cloning and characterization of a gene regulated by rIFN- $\gamma$  in U937 cells as well as in human mononuclear cells, fibroblasts and endothelial cells. Messenger RNA from this gene is induced within 30 min of rIFN- $\gamma$  treatment and demonstrates maximal (>30-fold) accumulation within 5 h. Increased transcription is partly responsible for this accumulation. This gene encodes a protein of relative molecular mass ( $M_r$ ) 12,378 which has significant amino-acid homology to platelet factor-4 and  $\beta$ -thromboglobulin, two chemotactic proteins released by platelets on degranulation. This IFN- $\gamma$ -inducible protein may be a member of a family of proteins involved in the inflammatory process.

A complementary DNA library was constructed from poly(A)<sup>+</sup> RNA isolated from U937 cells treated with recombinant  $\gamma$ -interferon (18 h induction, 100 U ml<sup>-1</sup>) and cloned into the *Pst*I site of pBR322 (refs 16, 17). Approximately 10,000 recombinants were screened for rIFN- $\gamma$ -inducible sequences by differential colony hybridization using cDNAs prepared from rIFN- $\gamma$ -induced and control poly(A)<sup>+</sup> RNAs. One cDNA clone,

pIFN- $\gamma$ -31, showed a dramatic induction by Northern blot analysis and was chosen for further characterization.

rIFN- $\gamma$  induces the accumulation of pIFN- $\gamma$ -31 mRNA in a dose-dependent manner. Figure 1a shows the accumulation of pIFN- $\gamma$ -31 mRNA in U937 cells in response to an 18-h incubation with various concentrations of rIFN- $\gamma$  and rIFN- $\beta$ . A detectable increase in pIFN- $\gamma$ -31 mRNA levels was seen at 10 U ml<sup>-1</sup> rIFN- $\gamma$ , with maximal accumulation at 100 U ml<sup>-1</sup> rIFN- $\gamma$ . pIFN- $\gamma$ -31 mRNA seems to be preferentially modulated by rIFN- $\gamma$ ; 100 times more antiviral units of rIFN- $\beta$  than rIFN- $\gamma$  were needed to produce a comparable effect (Fig. 1a, compare 10 U ml<sup>-1</sup> rIFN- $\gamma$  and 1,000 U ml<sup>-1</sup> rIFN- $\beta$ ). Even at very high doses of rIFN- $\beta$  (5,000 U ml<sup>-1</sup>), the accumulation of pIFN- $\gamma$ -31 mRNA was only 40% of the maximal accumulation seen with 100 U ml<sup>-1</sup> rIFN- $\gamma$ . The submaximal stimulation of pIFN- $\gamma$ -31 mRNA by rIFN- $\beta$  may result from cross-reactivity of this molecule with the IFN- $\gamma$  receptor at very high IFN- $\beta$  levels<sup>18</sup>.

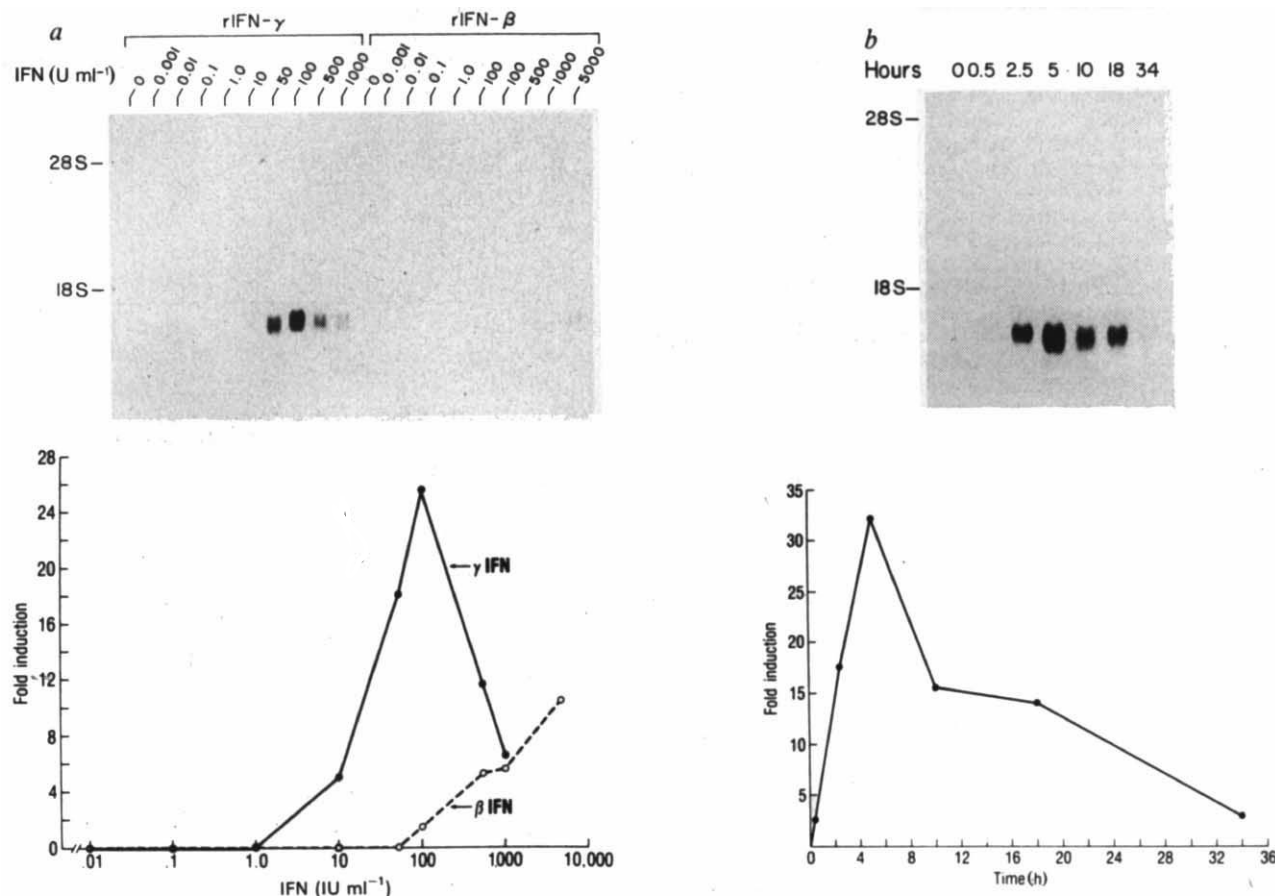
Figure 1b shows the kinetics of pIFN- $\gamma$ -31 mRNA accumulation at optimal rIFN- $\gamma$  concentration (100 U ml<sup>-1</sup>, see Fig. 1a). mRNA levels doubled within 30 min of induction and reached a maximal 34-fold increase within 5 h. By 18 h, half-maximal levels were observed, decreasing to a two-fold level by 34 h. In addition, 50  $\mu$ g ml<sup>-1</sup> of cycloheximide, which inhibited protein synthesis by >97%, did not inhibit the accumulation of pIFN- $\gamma$ -31 mRNA (data not shown). The rapidity with which pIFN- $\gamma$ -31 mRNA accumulates after rIFN- $\gamma$  induction and its insensitivity to protein synthesis inhibitors suggest that this is a primary response gene in IFN- $\gamma$  induction<sup>19</sup>.

To determine whether IFN- $\gamma$  affects transcription, the nuclear run-on transcription assay on isolated nuclei was used to measure the level of transcription of pIFN- $\gamma$ -31 mRNA in resting and rIFN- $\gamma$ -treated cells. Figure 1c demonstrates that rIFN- $\gamma$  increases the rate of transcription of pIFN- $\gamma$ -31 mRNA, thus establishing a role for  $\gamma$ -interferon in transcriptional activation. Recent studies<sup>20,21</sup> have demonstrated that the  $\alpha/\beta$ -interferons can affect the transcription of several genes within minutes after the binding of interferon to its receptor. The analysis of an IFN- $\alpha$ -responsive gene from neuroblastoma cells suggests that IFN- $\alpha$  is also regulating post-transcriptional events<sup>20</sup>.

To elucidate the function of pIFN- $\gamma$ -31, we determined the inducibility of this mRNA in different human cells. Figure 2 shows a panel of paired RNAs using various human cell lines, human peripheral blood mononuclear cells (PBM) and normal human spleen. To date, we have observed no primary cells which express this gene in the absence of rIFN- $\gamma$  induction, in contrast

to DR- $\alpha$  and HLA-B7, two molecules shown to be induced by IFN- $\gamma$ , which are constitutively expressed in many cells. pIFN- $\gamma$ -31 mRNA was preferentially induced in a primary human foreskin fibroblast after an 18-h incubation with 100 U ml<sup>-1</sup> rIFN- $\gamma$ . Human umbilical cord vein endothelial cells expressed pIFN- $\gamma$ -

31 mRNA after incubation with rIFN- $\gamma$  (100 U ml<sup>-1</sup>, 12 h), but not rIFN- $\beta$  (100 U ml<sup>-1</sup>, 12 h) (data not shown). Figure 2 also demonstrates that pIFN- $\gamma$ -31 mRNA is induced in PBM and U937 cells within 5 h of treatment with 100 U ml<sup>-1</sup> rIFN- $\gamma$ , again in contrast to the regulation of DR- $\alpha$  and HLA-B7 mRNAs in

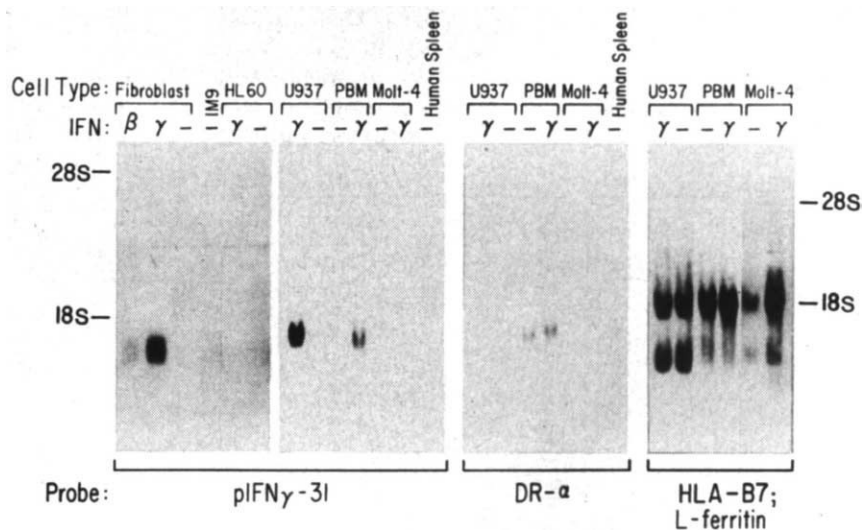


**Fig. 1** Dose response, kinetics and transcription of pIFN $\gamma$ -31 mRNA in response to IFN. **a**, Dose response of pIFN $\gamma$ -31 mRNA accumulation in response to interferons; **b**, kinetics of accumulation; **c**, nuclear run-on transcriptional analysis. **a**, Top, autoradiogram of RNA blot analysis of the accumulation of pIFN $\gamma$ -31 mRNA in U937 cells in response to 18-h incubations with various concentrations of rIFN- $\gamma$  and rIFN- $\beta$ . Bottom, quantitation of the RNA blot shown in the top panel by densitometer scanning (fold induction = densitometer units induced/densitometer units uninduced). **b**, Top, autoradiogram of the time course of pIFN $\gamma$ -31 mRNA accumulation in U937 cells in response to 100 U ml<sup>-1</sup> rIFN- $\gamma$ , with quantitation shown in the bottom panel. 28S and 18S ribosomal RNA size markers are indicated in both upper panels. **c**, Plasmids containing the indicated genes were bound to nitrocellulose filters and hybridized with <sup>32</sup>P-labelled run-on transcripts from nuclei isolated at 3.5 h after addition of 100 U ml<sup>-1</sup> rIFN- $\gamma$  to U937 cells. To quantify the transcriptional induction, the hybridizing signals were excised and counted by liquid scintillation. The relative rates of transcription are expressed as parts per million (p.p.m.). p.p.m. = (c.p.m. sample - c.p.m. control)/total input c.p.m. C, Control (uninduced);  $\gamma$ , rIFN- $\gamma$  induced.

**Methods.** **a**, **b**, The human histiocytic lymphoma cell line U937<sup>14</sup> was grown in RPMI-1640 supplemented with 10% fetal bovine serum. The recombinant interferons produced in *Escherichia coli* were generously provided by Hoffmann-La Roche. All the interferons were standardized by Hoffmann-La Roche using a cytopathic effect inhibition assay with vesicular stomatitis virus in WISH (HeLa) cells; activity was expressed in international antiviral units (U) per ml. The rIFN- $\gamma$  lot used had a specific activity of  $6.3 \times 10^6$  U mg<sup>-1</sup> and the rIFN- $\beta$  was clinical-grade material with a specific activity of  $8 \times 10^7$  U mg<sup>-1</sup>. The cells were induced with the interferons for this and all subsequent experiments at  $2.5 \times 10^5$  cells ml<sup>-1</sup>. RNA was isolated by the guanidine thiocyanate method<sup>38</sup> and 10  $\mu$ g per line was fractionated on a 1% agarose/2.2 M formaldehyde gel and transferred to nitrocellulose as described elsewhere<sup>39</sup>. The filters were hybridized with <sup>32</sup>P-nick-translated pIFN $\gamma$ -31.0 cDNA insert at a specific activity of  $2.5 \times 10^8$  c.p.m. per  $\mu$ g, washed in stringent conditions (0.1  $\times$  SSC, 0.1% SDS, 52  $^{\circ}$ C), then subjected to autoradiography with Kodak XAR film using a Dupont Cronex intensifying screen at -70  $^{\circ}$ C for 2.5 h. **c**, RNA chain elongation in isolated nuclei was performed as described elsewhere<sup>40</sup>, except that unincorporated nucleotide was removed by gel filtration of the purified nuclear RNA<sup>41</sup>.  $10^8$  nuclei were resuspended in 200  $\mu$ l of incubation buffer containing 5 mM dithiothreitol, 5 mM MgCl<sub>2</sub>, 90 mM KCl, 50% glycerol, 20 mM HEPES pH 7.6, 1 mM GTP, 1 mM CTP, 3 mM ATP, 250  $\mu$ Ci of <sup>32</sup>P-UTP, and incubated for 10 min at 37  $^{\circ}$ C. RNA purification was performed as described previously<sup>40</sup>; DNase I digestion was followed by deproteinization with phenol, chloroform/isoamyl alcohol extractions, and concentration by ethanol precipitation. Approximately 2  $\mu$ g of recombinant plasmid were linearized with *Bam*HI, boiled for 10 min, base denatured, neutralized and applied to 0.45- $\mu$ m nitrocellulose filters<sup>42</sup> using a Minifold dot apparatus (Schleicher & Schuell).  $6.9 \times 10^6$  c.p.m. ml<sup>-1</sup> and  $5.6 \times 10^6$  c.p.m. ml<sup>-1</sup> of trichloroacetic acid-precipitable counts were used in rIFN- $\gamma$ -induced and control hybridization reactions, respectively. Hybridization, RNase A and T<sub>1</sub> digestion, and washing of filters were performed as described previously<sup>40</sup>. The X-ray film was exposed at -70  $^{\circ}$ C with an intensifying screen for 4 days. The HLA-B7 cDNA plasmid was generously provided by S. Weissman<sup>43</sup>.



**Fig. 2** Cell distribution and IFN- $\gamma$  dependence of pIFN $\gamma$ -31 mRNA accumulation. RNA blots were performed on the RNAs extracted from the indicated cells induced with rIFN- $\gamma$  ( $\gamma$ ) or induced with rIFN- $\beta$  ( $\beta$ ), or which received no treatment (-). Filters were hybridized with the probe indicated below the blots. **Methods.** RNA transfers contained U937, PBM, MOLT-4 and normal human spleen RNA. RNA (10  $\mu$ g per lane) was fractionated; the 18S and 28S ribosomal RNAs are indicated on the far right. In the blot probed with HLA-B7 and L-ferritin, the HLA-B7 mRNA is the larger of the two transcripts. The far-left blot contains fibroblast, HL60 and IM9 RNA. In this blot, 2  $\mu$ g of poly(A)<sup>+</sup> RNA was fractionated per lane; the 18S and 28S rRNA for this filter are indicated on the far left. U937 cells, PBM and MOLT-4 cells were induced with 100 U ml<sup>-1</sup> rIFN- $\gamma$  for 5 h. The fibroblasts and HL-60 cells were induced with 100 U ml<sup>-1</sup> of both rIFN- $\beta$  and rIFN- $\gamma$  for 18 h. RNA isolation, RNA blot analysis and interferon induction were performed as indicated in Fig. 1 legend, except that the fibroblasts were confluent monolayers. The primary human foreskin fibroblast FS4 was obtained from S. Gupta and grown in minimum essential medium supplemented with 10% fetal calf serum (FCS). The cell lines HL60, IM9 and MOLT-4 were grown in RPMI-1640 supplemented with 10% FCS. Normal human peripheral blood was fractionated on a Ficoll-Hypaque gradient and the PBM were cultured in RPMI-1640 + 10% FCS. Normal human spleen was obtained from an incidental splenectomy and RNA was isolated by the guanidine thiocyanate method<sup>38</sup>. The HLA-DR  $\alpha$ -chain cDNA plasmid was generously provided by J. Lee<sup>44</sup> and the human L-ferritin cDNA plasmid by M. H. Dorner<sup>45</sup>.

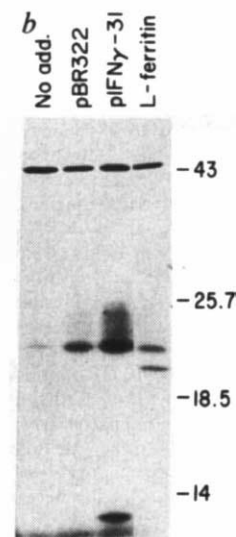


PBM and U937 cells where, 5 h after rIFN- $\gamma$  stimulation, no significant modulation in mRNA levels was detected. When cells from the human T-cell leukaemia cell line MOLT-4 were incubated for 18 h with 100 U ml<sup>-1</sup> rIFN- $\gamma$ , an increased accumulation of HLA-B7 mRNA was observed, but no expression of either DR- $\alpha$  mRNA or pIFN $\gamma$ -31 mRNA. HL-60 cells, a promyelocytic leukaemia cell line, showed no induction of pIFN $\gamma$ -31 mRNA after an 18-h incubation with 100 U ml<sup>-1</sup> rIFN- $\gamma$ . Induction of pIFN $\gamma$ -31 mRNA by rIFN- $\gamma$  in mononuclear cells, fibroblasts and endothelial cells indicates that this gene is induced in a diverse population of cells in response to IFN- $\gamma$ .

The DNA sequence of pIFN $\gamma$ -31 was obtained from two overlapping cDNA clones (Fig. 3a). Each base was sequenced from both strands using the dideoxy method of Sanger and Coulson<sup>22</sup> and the chemical cleavage method of Maxam and Gilbert<sup>23</sup>. The longest open reading frame begins at nucleotide 70, extending for 294 nucleotides before terminating at nucleotides 364-366 with TAA. pIFN $\gamma$ -31 mRNA contains the classical polyadenylation signal AAUAAA, 12 bases from the start of the poly(A) tail (underlined in Fig. 3a). There is a long 811-base 3'-untranslated region, a 294-nucleotide coding region and a ~220-nucleotide 5'-untranslated region.

**Fig. 3** Primary structural analysis of pIFN $\gamma$ -31 cDNA clones. **a**, Determined nucleotide sequence and predicted amino-acid sequence of pIFN $\gamma$ -31; **b**, hybridization selection and translation of pIFN $\gamma$ -31 mRNA. The coding strand is displayed in **a**; the number of the nucleotide residue at the right end of each line is given. The polyadenylation signal is underlined. The deduced amino-acid sequence is shown above the nucleotide sequence, numbered beginning with the putative initiating methionine. The hydrophobic core of the predicted signal sequence is overlined and the predicted signal peptidase cleavage site is indicated by an arrow. Hybridization selection and translation of pIFN $\gamma$ -31 mRNA (**b**) were performed to confirm the open reading frame predicted above. Poly(A)<sup>+</sup> mRNA from U937 cells induced by 5 h treatment with rIFN- $\gamma$  (100 U ml<sup>-1</sup>) was annealed to nitrocellulose filters containing pIFN $\gamma$ -31 and control clones pBR322 and L-ferritin. The mRNA was eluted and translated *in vitro* using a rabbit reticulocyte lysate extract and <sup>35</sup>S-methionine label. An autoradiogram of the translation products fractionated on a 12.5% SDS-polyacrylamide gel is shown in **b**. The 'no add.' lane shows only the endogenous translation products of the extract. *M<sub>r</sub>* markers are displayed on the right.

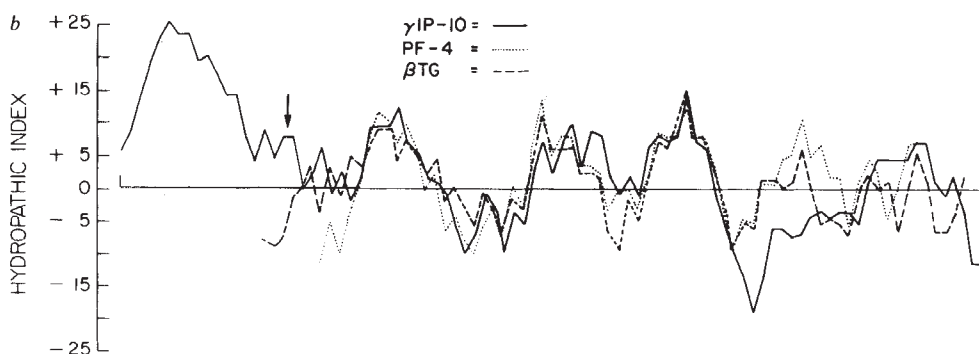
**a** CCGGAGACATTCTCTCAATTGCTTAGACATATCTTGAGCCTACAGCAGGGAACCTCCAGTCTCAGCACC 69  
 1 Met Asn Gln Thr Ala Ile Leu Ile Cys Cys Leu Ile Phe Leu Thr Leu Ser Gly 123  
 ATG AAT CAA ACT GCG ATT CTG ATT TGC TGC CTT ATC TTT CTG ACT CTA AGT GGC  
 19 Ile Gln Gly Val Pro Leu Ser Arg Thr Val Arg Cys Thr Cys Ile Ser Ile Ser 177  
 ATT CAA GGA GTA CCT CTC TCT AGA ACC GTA GCG TGT ACC TGC ATC AGC ATT AGT  
 37 Asn Gln Pro Val Asn Pro Arg Ser Leu Glu Lys Leu Glu Ile Ile Pro Ala Ser 231  
 AAT CAA CCT GTT AAT CCA AGG TCT TTA GAA AAA CTT GAA ATT ATT CCT GCA AGC  
 55 Gln Phe Cys Pro Arg Val Glu Ile Ile Ala Thr Met Lys Lys Lys Gly Glu Lys 285  
 CAA TTT TGT CCA CGT GTT GAG ATC ATT GCT ACA ATG AAA AAG AAG GGT GAG AAG  
 73 Arg Cys Leu Asn Pro Glu Ser Lys Ala Ile Lys Asn Leu Leu Lys Ala Val Ser 339  
 AGA TGT CTG AAT CCA GAA TCG AAG GCC ATC AAG AAT TTA CTG AAA GCA GTT AGC  
 91 Lys Glu Met Ser Lys Arg Ser Pro 98  
 AAG GAA ATG TCT AAA AGA TCT CCT TAAACACGAGGGGAGCAAAATCGATGCACTGCTCCAA  
 GGATGGACACACAGAGGCTGCTCTCCATCACTTCTCCATCATGAGTATATGTCAGGCATAATTGTC 473  
 TTATTTTCAGTTTACTACTAAAGGTGACCAATGATGTCACCAATCACTACTACTCTGCTGAGGAAG 544  
 GTTAATGTCATCATCTCAAGCTATTAGTAACTCTACCTGGCATAATAATGTAAGCTCTACTGAGG 615  
 TGCTATGTTCTTAGTGGATGTTCTGACCTGCTTCAAAATTTCCCTCACCTTTCCCATCTTCAAGGGA 686  
 CTAGGAATCTTTCTGCTTTGGGTTTATCAGAAATCTCAGAAATCTCAAAATCAAAAGGATGATGCAATCA 757  
 AATCTGCTTTTAAAGAAATGCTCTTACTTCTAGGACTTCACTGCACTCTCCCAAGGGGCCAAATCT 828  
 TTCAGTGGCTACCTACATCAATCCAAACACATACAGGAAGGTAGAAATATCTGAAATGATGTTGTAAG 899  
 TATCTTATTTAATGAAGACATGACAAAGTATAGTCTTAGATGATATATTTCTATATTTCTTCACT 970  
 GTACATGGAATCACTGATTAATGATCTATGATCAATGAGTAAACAGGAAATTTTAAATAACAGATCA 1041  
 ATATATGCTCTGATGTTTACATGAAGATAAATGCTGAATGGTTTCAATATAAAATGAGGTACTCTCTG 1112  
 GAAATATTAAGAAAGACTCTTAATGTTGAAGTCAAGGTTAATAAGTAAATATAAATAAATAAATAA 1183  
 AAAAAAAAAAAAAA



**Methods.** **a**, DNA sequence analysis was performed using the dideoxy method of Sanger and Coulson<sup>22</sup> and the chemical cleavage method of Maxam and Gilbert<sup>23</sup>. The entire coding region of pIFN $\gamma$ -31 consisted of two overlapping cDNAs. pIFN $\gamma$ -31.0 was isolated in the differential colony hybridization screening, extended from nucleotides 451 to 1,198, and used to rescreen a size-fractionated cDNA library. 10,000 recombinants were screened with an *Eco*RI/*Pvu*III fragment of pIFN $\gamma$ -31.0. Seven colonies hybridizing to this probe were picked, rescreened, then analysed by restriction mapping. One clone, pIFN $\gamma$ -31.7, contained the most 5'-nucleotide sequences and extended from nucleotides 1 to 875. **b**, Hybridization selection of specific mRNAs was performed essentially as described previously<sup>46</sup>. Plasmid DNA (10  $\mu$ g) were linearized and bound to 0.45- $\mu$ m nitrocellulose filters as indicated in Fig. 1 legend. Hybridization was carried out at 50 °C for 3 h in a 250- $\mu$ l volume containing 65% formamide, 20 mM PIPES (pH 6.4), 0.1% SDS, 0.4 M NaCl, 50  $\mu$ g yeast transfer RNA and 45  $\mu$ g poly(A)<sup>+</sup> RNA. Filters were then washed six times with 50 ml of 0.15 M NaCl, 0.01 M Tris pH 7.6, 1 mM EDTA, 0.5% SDS at 65 °C. The filters were washed twice more with 50 ml 0.15 M NaCl, 0.01 M Tris pH 7.6, 1 mM EDTA at 65 °C. Selected RNAs were eluted by boiling in 300  $\mu$ l of H<sub>2</sub>O for 60 s and then quickly freezing in a dry-ice ethanol bath. The eluted RNAs were extracted with CHCl<sub>3</sub>/phenol and then ethanol precipitated in 0.25 M sodium acetate with 20  $\mu$ g yeast tRNA as a carrier. The pellets were washed once with 70% ethanol and dissolved in 5  $\mu$ l H<sub>2</sub>O. Each selected RNA (1  $\mu$ l) was used to program an Amersham RRL *in vitro* translation system using <sup>35</sup>S-methionine as label.

[illegible]

**Fig. 4** *a*, Amino-acid homology between  $\gamma$ IP-10, PF-4-human (PF-4), PF-4-bovine (PF4 30) and  $\beta$ -TG-human ( $\beta$ TG-Hu). The optimized amino-acid alignment, which includes gaps, was done by implementing a Needleman-Wunsch algorithm<sup>47</sup> over the initial dfastp alignment. The one-letter amino-acid notation is used. A colon indicates an exact match, a period a 'conservative mutation' (can be obtained with only one DNA base change) and a blank a 'non-conservative mutation' (at least two DNA bases would have to be changed). *b*, Hydropathy profile of  $\gamma$ IP-10 (—), PF4-Hu (···) system of Kyte and Doolittle<sup>48</sup>. The  $i+3$ . Positive values indicate hydrophobicity. The arrow indicates the N-terminal carboxyl terminus (left to right along the sequence). The hydropathy profiles are overlaid to obtain the composite seen in panel *c*.



The deduced amino-acid sequence (Fig. 3a) beginning with the initiation codon AUG at nucleotides 70–72 encodes a protein of 98 amino acids and predicts a protein of  $M_r$  12,378, termed  $\gamma$ IP-10. The highly hydrophobic stretch of 10 amino acids (residues 5–14 overlined in Fig. 3a) is typical of the hydrophobic core of a signal peptide<sup>24</sup>. Application of the empirical rules proposed by Von Heijne<sup>25</sup> predicts the signal peptidase cleavage site to be after residue 21 (Gly, arrow Fig. 3a). Analysis of the amino-acid sequence reveals no other hydrophobic regions which could serve as a transmembrane region and anchor the protein in the membrane. This suggests that the protein is secreted and that the unmodified mature protein has a  $M_r$  of 9,979. Consistent with  $\gamma$ IP-10 containing a signal peptide, its mRNA is enriched in the microsomal fraction (data not shown). The predicted amino-acid sequence for  $\gamma$ IP-10 contains no *N*-linked glycosylation sites (Asn-X-Thr/Ser).

Hybridization selection and translation of pIFN- $\gamma$ -31 were performed to confirm the predicted open reading frame. Poly(A)<sup>+</sup> mRNA from U937 cells treated for 5 h with rIFN- $\gamma$  was annealed to nitrocellulose filters containing pIFN- $\gamma$ -31 and control clones (L-ferritin and pBR322) and then eluted. The selected mRNA was translated using a rabbit reticulocyte lysate with <sup>35</sup>S-methionine label and fractionated on a 12.5% SDS-polyacrylamide gel (Fig. 3b). mRNA from clone pIFN- $\gamma$ -31 directs the synthesis of a ~12,000-*M<sub>r</sub>* protein which is not present in the control lanes, confirming the existence of an mRNA which can direct the synthesis of the  $\gamma$ IP-10 protein in rIFN- $\gamma$ -induced U937 cells.

Using the program 'dfastp' (ref. 26), we searched the National Biomedical Research Foundation Library for any protein containing homology to the predicted amino-acid sequence for  $\gamma$ IP-10. Two proteins, platelet factor-4 (PF-4) and  $\beta$ -thromboglobulin ( $\beta$ -TG), have significant homology to the predicted amino-acid sequence of  $\gamma$ IP-10. Alignment of these three sequences is shown in Fig. 4a.  $\gamma$ IP-10, PF-4 and  $\beta$ -TG demonstrate 35% identity in a 62-amino-acid overlap.  $\beta$ -TG has been reported to contain two disulphide bonds linking residues 16-42 and 18-58 (ref. 27). When PF-4,  $\beta$ -TG and  $\gamma$ IP-10 were aligned for maximum homology (Fig. 4a), the cysteine residues of the mature proteins were perfectly aligned, suggesting preservation of tertiary structure among these molecules. Consistent with this notion is the similarity in the hydropathy profile of the proteins (Fig. 4b); there is a striking conservation of hydrophobic (peaks) and hydrophilic (troughs) domains between the three proteins.

The hydrophobic core of the predicted leader peptide of  $\gamma$ IP-10 is clearly evident with the predicted signal peptidase cleavage site indicated by an arrow in Fig. 4b.

PF-4 and  $\beta$ -TG proteins are stored in the  $\alpha$  granule of platelets and secreted concomitantly during the platelet-release reaction<sup>28</sup>. Although the *in vivo* functions of PF-4 and  $\beta$ -TG remain unclear, *in vitro* studies suggest that these proteins are important mediators of inflammation and wound healing<sup>29-37</sup>. The homology between  $\gamma$ IP-10, PF-4 and  $\beta$ -TG suggests that  $\gamma$ IP-10 may also be involved in the inflammatory response. The rapid and specific induction of pIFN $\gamma$ -31 mRNA by IFN- $\gamma$  in a diverse population of cells suggests that  $\gamma$ IP-10 may be central to the IFN- $\gamma$  response. The role for this family of related polypeptides in inflammation and immune cell regulation as well as the molecular characterization of transcriptional activation by IFN- $\gamma$  are under investigation.

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**Table 1** Characteristics of mice used (a) and binding capacity of partially purified insulin receptor preparations (b)

| a                                   | Lean                                         | Obese       |
|-------------------------------------|----------------------------------------------|-------------|
| Body weight (g)                     | 43.4 ± 1.2                                   | 66.5 ± 3.5  |
| Blood glucose (mg per 100 ml)       |                                              |             |
| Before insulin                      | 162 ± 8                                      | 334 ± 27    |
| 30 min after insulin                | 61 ± 4                                       | 294 ± 37    |
| Insulinaemia (ng ml <sup>-1</sup> ) | 3.2 ± 0.7                                    | 50.8 ± 11.1 |
| b                                   | Specific binding of <sup>125</sup> I-insulin |             |
|                                     | Lean                                         | Obese       |
| Soleus muscles                      | 0.52 ± 0.04                                  | 0.37 ± 0.03 |
| (per cent per mg protein)           |                                              |             |
| Partially purified receptors        | 3.81 ± 0.21                                  | 2.94 ± 0.22 |
| (per cent per µg protein)           |                                              |             |

Mice were injected with gold thioglucose at 3 weeks of age and used at 25-30 weeks<sup>3,4</sup>. Insulin resistance was tested *in vivo* by measuring plasma glucose before and after an intravenous insulin injection (0.5 U per kg body weight). Insulin binding was measured in soleus muscles as described previously<sup>3</sup> or in partially purified insulin receptors: 10 µl of partially purified receptor preparations was incubated in HEPES (50 mM), NaCl (150 mM) buffer pH 7.6 containing <sup>125</sup>I-labelled insulin (10<sup>-11</sup> M) in the absence or presence of unlabelled insulin (10<sup>-6</sup> M) in a final volume of 200 µl. After 2 h at 20 °C, bound insulin was separated from unbound hormone using polyethylene glycol. Nonspecific binding represented 10-15% of total binding. Values are the mean ± s.e.m. of five measurements for insulin test, eight for soleus muscles or six for partially purified receptor preparations.

## Insulin receptor tyrosine kinase is defective in skeletal muscle of insulin-resistant obese mice

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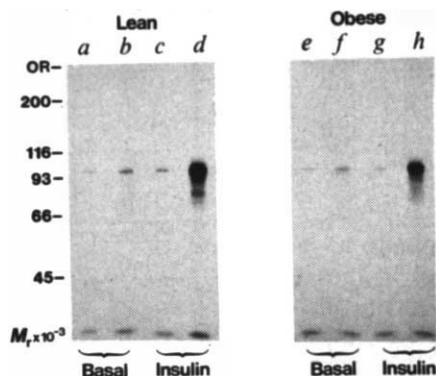
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Obese syndromes of genetic origin or experimentally induced are characterized by resistance to insulin both *in vivo* (association of hyperglycaemia and hyperinsulinaemia) and *in vitro*<sup>1</sup>. Thus, skeletal muscle of obese mice, which is the most important target organ for the action of insulin, displays a reduced response to insulin<sup>2-4</sup>. This hormonal resistance cannot be explained by the moderate decrease in the number of insulin receptors found in obese animals<sup>3-5</sup>. In fact, it is generally believed that a biochemical event occurring very early after binding of insulin to its receptor, which is the first step in insulin action, is defective in obesity. One of the earliest post-binding events so far recognized, and which is thought to have a key role in cellular signalling by the insulin receptor, is the insulin-stimulated phosphorylation of its receptor<sup>6-8</sup>. In an effort to localize the defect responsible for the insulin resistance in obesity, we have studied the insulin receptor protein kinase activity and we show here that insulin receptors from skeletal muscles of insulin-resistant obese mice have an altered kinase activity for phosphorylation of both the receptor itself and of exogenous substrates.

The animals used in the present study were male Swiss albino mice. Mice were rendered obese with a double injection of gold thioglucose at 3 weeks of age and they were used at 25-30 weeks when obesity was fully expressed<sup>3,4</sup>. Lean controls were age- and sex-matched animals. All animals were fed *ad libitum* on a standard diet until the time of killing. Following gold thioglucose injection, which induces hyperphagia, mice became markedly obese (Table 1). They were hyperglycaemic and insulin-resistant since insulin induced *in vivo* a poor hypoglycaemic effect. Using wheat-germ lectin chromatography, partially

purified insulin receptors were prepared from skeletal muscle of lean and obese mice, and they were then used in a cell-free phosphorylation assay as described previously<sup>7-9</sup>. For all tissues studied so far, insulin enhances several-fold the phosphorylation of its receptor  $\beta$ -subunit (relative molecular mass,  $M_r$ , 95,000); the other receptor subunit, termed  $\alpha$ -subunit ( $M_r$ , 130,000) and containing the insulin binding site, is generally not phosphorylated<sup>10</sup>. Similar results were found with receptors derived from rat skeletal muscle<sup>11</sup>. With insulin receptors obtained from skeletal muscle of lean mice, insulin markedly stimulated the phosphorylation of a polypeptide of  $M_r$  95,000 (Fig. 1); this phosphoprotein corresponds to the  $\beta$ -subunit of the insulin receptor, as it was immunoprecipitated by a highly specific antiserum<sup>12</sup> to insulin receptor (lanes d, h), but not by normal serum (lanes c, g). More importantly, the effect of insulin on phosphorylation of its receptor was less pronounced in the preparation from insulin-resistant obese mice (lane h) compared with lean controls (lane d). The decreased insulin effect observed in obese mice is unlikely to result from a residual occupation of insulin receptors due to the high levels of circulating insulin in obese mice (Table 1), as basal phosphorylation was negligible in both groups of mice (lanes b, f).

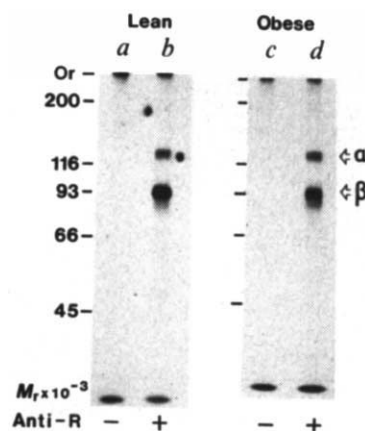
Insulin binding was decreased only to a moderate extent (~30%) in intact soleus muscles<sup>3</sup> from obese mice compared with controls, and a similar moderate defect (22%) in insulin binding was observed using partially purified insulin receptors from obese animals (Table 1). Scatchard analysis of the insulin binding data revealed that this was due only to a decrease in receptor number without any change of binding affinity (data not shown). The altered autophosphorylation of insulin receptors from skeletal muscle of obese mice could be due to a decreased number of receptors or, alternatively, due to an intrinsic defect of the insulin receptor kinase in obese mice. To approach this question, we next studied insulin receptors from skeletal muscle of lean and obese mice for self-phosphorylation of the receptor  $\beta$ -subunits present in identical amounts of receptors, as measured by Scatchard analysis and affinity labelling of  $\alpha$ -subunits with <sup>125</sup>I-insulin. Partially purified insulin receptors were incubated with <sup>125</sup>I-photoreactive insulin followed by ultraviolet irradiation for covalent affinity labelling of insulin receptors. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) revealed the presence of a major labelled polypeptide with  $M_r$  130,000, which we recognize as the insulin receptor



**Fig. 1** Phosphorylation of skeletal muscle insulin receptors from lean and insulin-resistant obese mice. Skeletal muscles were obtained from the hindlegs of 30-week-old normal Swiss mice (lean) or mice injected with gold thioglucose (obese). Muscles (3 g) were homogenized (15 s, Polytron homogenizer, 2,000 r.p.m.) in 3 vol. HEPES (50 mM, pH 7.6), NaCl (150 mM), bacitracin (1 mM), aprotinin (1,000 trypsin-inhibitor units per ml), phenylmethylsulphonyl fluoride (1 mM) and Triton X-100 (1%). The homogenates were solubilized for 90 min at 4°C by continuous stirring, and centrifuged at 150,000g at 4°C for 90 min. The supernatants were applied to a wheat-germ-agglutinin-agarose column; after washing, bound glycoproteins were desorbed with 0.45 M *N*-acetylglucosamine<sup>9</sup>. Preparations from lean and obese mice were always performed in parallel. Partially purified insulin receptors were incubated in a reaction mixture containing HEPES (50 mM, pH 7.6), NaCl (150 mM) without (basal) or with 0.1  $\mu$ M insulin at 15°C for 90 min. Thereafter, the phosphorylation reaction was initiated adding [ $\gamma$ -<sup>32</sup>P]ATP (15  $\mu$ M), MnCl<sub>2</sub> (4 mM) and MgCl<sub>2</sub> (8 mM). After 30 min at 20°C, the reaction was stopped by adding ice-cold stopping solution containing NaF (80 mM) and EDTA (30 mM). Insulin receptors were specifically immunoprecipitated with antiserum containing autoantibodies to the insulin receptor<sup>12</sup> (serum from patient B2 at a 1:300 dilution) (lanes b, d, f, h) or normal serum (lanes a, c, e, g) after a preclearing step with normal serum. The immunoprecipitates were boiled for 5 min in a solution containing SDS (3%, w/v), glycerol (10%, v/v), sodium phosphate (10 mM), 2-mercaptoethanol (5%) and bromophenol blue (0.05%) and subjected to one-dimensional SDS-PAGE with a 7.5% acrylamide resolving gel<sup>21</sup>. An autoradiograph of the gel is shown.

$\alpha$ -subunit for the following reasons. First, the radioactive band with  $M_r$  130,000 was not found when unlabelled insulin ( $10^{-7}$  M) was present together with <sup>125</sup>I-photoreactive insulin during the binding step. Second, the 130,000- $M_r$  polypeptide was immunoprecipitable by anti-insulin antiserum and anti-receptor antiserum, but not by normal serum (data not shown). Based on estimates derived from Scatchard analysis of insulin binding, we took identical amounts of insulin receptors partially purified from skeletal muscle of lean mice and from insulin-resistant obese mice, respectively. Then, <sup>125</sup>I-photoreactive insulin was first covalently attached to the two receptor preparations, and the receptors were used in a cell-free phosphorylation assay as described previously. Finally, insulin receptors were specifically immunoprecipitated with antibodies to insulin receptor. The autoradiogram in Fig. 2 clearly shows that for an equal amount of insulin binding, as reflected by the identical labelling intensity of the receptor  $\alpha$ -subunit, a much lower degree of phosphorylation of the insulin receptor  $\beta$ -subunit is found in partially purified receptors obtained from obese mice compared with lean mice. Thus, when the radioactivity present in the insulin receptor  $\alpha$ - and  $\beta$ -subunits was measured by scanning densitometry in three different experiments, and the ratio of labelling of the  $\beta$ -subunit over the  $\alpha$ -subunit was calculated, we found  $2.9 \pm 0.3$  in lean mice, and  $1.7 \pm 0.2$  in obese mice (mean  $\pm$  s.e.m. of three experiments, each performed with a different insulin receptor preparation). This result indicates that in obese mice there is a marked alteration in the insulin-induced phosphorylation of the insulin receptor.

To evaluate further the possible role of a defect in insulin receptor kinase activity in the resistance to insulin seen in

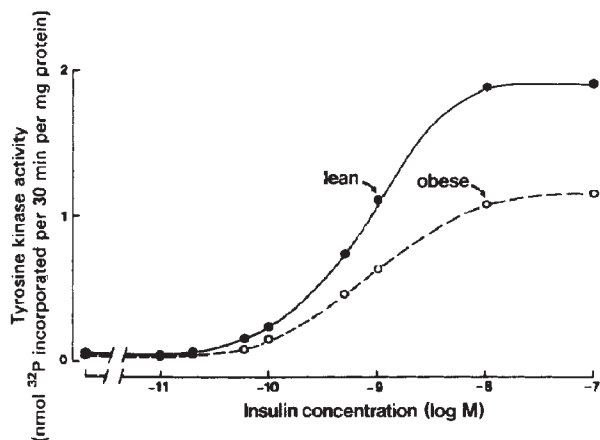


**Fig. 2** Double labelling of  $\alpha$ - and  $\beta$ -subunits of insulin receptors in lean and insulin-resistant obese mice. Partially purified insulin receptors were prepared from lean and obese mice as described in Fig. 1 legend. The amount of insulin receptors in the two preparations was determined by Scatchard analysis of insulin binding data; obese mice showed a reduction of 20% compared with lean mice (there was no change in insulin binding affinity). Identical amounts of receptors from lean and obese mice were first incubated with 5 nM <sup>125</sup>I-labelled photoreactive insulin (B29 (2-nitro,4-azido)phenylacetyl insulin) for 2 h at 15°C in the dark; samples were then irradiated for 5 min at 4°C using a high-pressure mercury lamp (Philips HPK; 125 W h<sup>-1</sup>) as previously described<sup>22</sup>. Using this procedure, 20% of photoreactive <sup>125</sup>I-insulin was covalently linked to solubilized receptors after ultraviolet irradiation. At the end of photoactivation, phosphorylation was carried out as detailed in Fig. 1 legend. The labelled insulin receptors were then exposed to normal serum (lanes a and c) or anti-receptor serum B2 (lanes b and d), and the immunoprecipitates were subjected to SDS-PAGE under reducing conditions. An autoradiogram of the gel is shown.

obesity, the ability of insulin receptors to catalyse the phosphorylation of exogenous substrates was investigated. The substrate used here was a copolymer of glutamate and tyrosine residues in a 4:1 ratio, which has been shown to be an excellent substrate for the insulin receptor protein kinase<sup>13</sup>. Insulin binding to the partially purified insulin receptors obtained from skeletal muscle of obese mice and used in these experiments was only decreased by 15% compared with lean mice. In contrast, phosphorylation of the substrate by partially purified insulin receptors from obese mice was decreased by 40% at all insulin concentrations compared with lean mice (Fig. 3). For both preparations, a half-maximal effect was obtained with  $8 \times 10^{-8}$  M insulin.

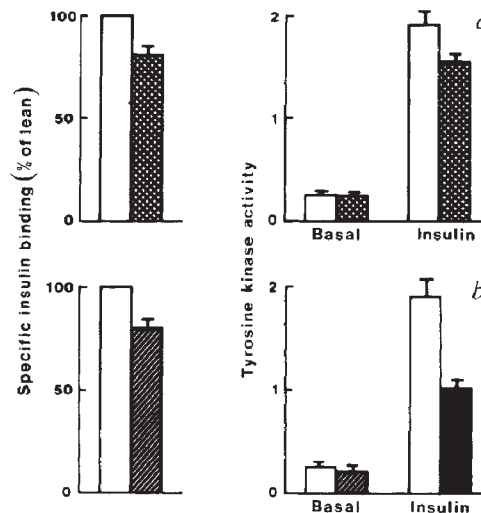
In our attempts to test the putative causal association between insulin resistance and loss of receptor kinase activity, we have investigated whether both defects evolve concomitantly in mice rendered obese. The insulin receptor tyrosine kinase activity of skeletal muscle was thus evaluated in young obese mice, which are not insulin-resistant, as they are hyperinsulinaemic and slightly hypoglycaemic, and they normally respond to exogenous insulin<sup>4</sup>. These 10-week-old non-insulin-resistant obese mice were compared with 30-week-old obese animals, which have developed an overt resistance to insulin. For both groups of obese mice, insulin binding to partially purified skeletal muscle insulin receptors was ~80% of the binding in the corresponding age-matched lean animals. In skeletal muscle from 10-week-old obese mice, the insulin-stimulated receptor tyrosine kinase activity towards an exogenous substrate was reduced in direct proportion to the decrease in insulin binding, that is, a reduction of approximately 20%. This is in striking contrast to the situation observed in 30-week-old insulin-resistant obese mice, where the diminution in receptor kinase activity (45%) is much more pronounced than would have been expected from the decrease in insulin binding (20%). Thus, in obesity with the development of insulin resistance and without any further change in insulin receptor number, a defect intrinsic to the insulin receptor tyrosine kinase activity appears.





**Fig. 3** Kinase activity of insulin receptors from lean and insulin-resistant obese mice towards an exogenous substrate. Partially purified insulin receptors (25  $\mu$ l), prepared from lean and obese mice as described in Fig. 1 legend, were preincubated in HEPES (50 mM), NaCl (150 mM), bovine serum albumin (0.2 mg ml<sup>-1</sup>) buffer, pH 7.6 (final volume 140  $\mu$ l) without or with various concentrations of insulin for 60 min at 24 °C. The substrate<sup>13</sup> (copolymer of glutamate/tyrosine, 4:1, 0.25 mg ml<sup>-1</sup>) was then added for an additional 30 min. The phosphorylation reaction was initiated by adding 20  $\mu$ l of a solution containing [ $\gamma$ -<sup>32</sup>P]ATP, MnCl<sub>2</sub> and MgCl<sub>2</sub> at final concentrations of 20  $\mu$ M, 3,000 c.p.m. pmol<sup>-1</sup>, 4 mM and 8 mM, respectively. After 30 min the reaction was stopped by applying 75- $\mu$ l samples to filter papers (Whatman ET31) and soaking the papers in a bath of 10% trichloroacetic acid (TCA), 10 mM sodium phosphate. After extensive washing in 5% TCA, radioactivity of the TCA-precipitable material was measured using Cerenkov radiation. Results are expressed as nmol of phosphate incorporated per mg receptor protein per 30 min.

Here we have presented evidence that the insulin receptor tyrosine kinase activity is markedly impaired in skeletal muscle of insulin-resistant obese mice. This defect in the enzyme function of the receptor was observed for both receptor autophosphorylation and the ability of the receptor to catalyse the phosphorylation of a synthetic substrate. This result indicates that the insulin-resistant state accompanying obesity can be related to an impaired activity of the insulin receptor kinase. Similarly, it has also been shown recently that the insulin resistance, which occurs in streptozotocin-diabetic rats, is accompanied by a decreased kinase activity of liver insulin receptors<sup>14</sup>. Our finding represents the earliest post-receptor binding alteration recognized so far in animal obesity, in which the insulin resistance is generally accepted to result mainly from a post-receptor defect. This observation may have important implications for our understanding of the insulin resistance commonly found in human obesity, which is the most frequent cause of hormone resistance in humans. Our model of animal obesity has a major feature in common with human obesity in that it is acquired. It thus excludes the possibility that the insulin receptor kinase activity is deficient due to the result of a genetic abnormality of the enzyme as it appears to occur in two recently reported cases of insulin resistance in extremely rare patients<sup>15,16</sup>. The alteration of intrinsic insulin receptor kinase activity in our obese animals could result from the chronic hyperinsulinaemia present in obese mice which induces a desensitized state of the receptor-associated enzyme function. Along this line, it has been suggested that diet-induced alterations of insulin receptor kinase activity could be related to different nutritional states or ambient plasma insulin levels in fasted and fasted, re-fed animals<sup>17</sup>. Our observations are similar to those of Carlin *et al.*<sup>18</sup> who show that senescent fibroblasts, which are resistant to epidermal growth factor (EGF) action, have a defect in their EGF receptor kinase despite an unchanged number of EGF receptors. Alternatively, the defective insulin receptor kinase activity found in obese mice could be due to inadequate coupling between the two insulin receptor subunits. The mechanism of signal transfer from the insulin binding subunit ( $\alpha$ ) to the  $\beta$ -subunit, responsible for



**Fig. 4** A defect in insulin receptor tyrosine kinase activity appears with insulin resistance in skeletal muscle from obese mice. Insulin receptors were partially purified from skeletal muscle obtained from the following two groups of lean (open bars) and obese (shaded bars) mice: *a*, mice were aged 10 weeks, and the obese animals were not insulin-resistant (body weight (g) 35.9  $\pm$  0.3 and 45.1  $\pm$  1.0; blood glucose (mg per 100 ml) 217  $\pm$  6 and 182  $\pm$  6; insulinaemia (ng ml<sup>-1</sup>) 1.9  $\pm$  0.4 and 7.7  $\pm$  1.8 for lean and obese mice, respectively; values are mean  $\pm$  s.e.m. of 8–12 values; all differences were statistically significant with  $P < 0.01$ . *b*, Mice were 30 weeks old and the obese animals were insulin-resistant as described in Table 1. Insulin binding was measured in partially purified insulin receptors as described in Table 1, and the results are expressed as a percentage of the binding obtained in preparations from lean mice. Insulin receptor tyrosine kinase activity towards an exogenous substrate was measured in the absence (basal) or presence of insulin ( $10^{-7}$  M) as described in Fig. 3 legend and is expressed in nmol <sup>32</sup>P incorporated per mg receptor protein per 30 min. Values are the mean of five experiments, each performed with a different insulin receptor preparation.

the kinase activity, is unknown. It could be envisaged that this signal transfer is hampered in insulin-resistant obese mice. In contrast, a quantitative defect of solely the insulin receptor  $\beta$ -subunit in obese mice is very unlikely. Indeed, insulin-induced receptor loss (a phenomenon known as down-regulation<sup>19</sup>) leads to an identical decrease in both insulin receptor subunits<sup>20</sup>. Along the same line, the insulin receptor of obese mice could have a greater proportion of nicked  $\beta$ -subunits than lean mice. However, a careful analysis of our gels does not favour this contention.

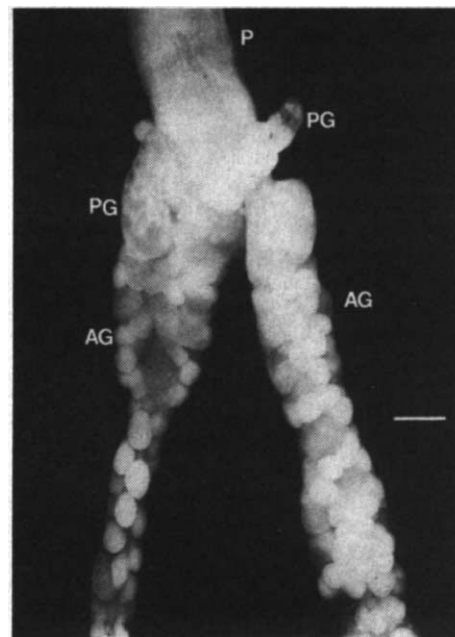
It is generally believed that protein phosphorylation is an important mechanism by which a variety of extracellular stimuli regulate cellular functions. For the insulin receptor, the hormone-stimulated phosphorylation of its receptor is a very early post-binding event, and a fundamental property of the receptor as it occurs in all tissues studied thus far. The demonstration of an altered insulin-stimulated kinase associated with the insulin receptor activity in muscles of obese mice, which display insulin resistance for a variety of parameters (glucose transport and metabolism, glycogen synthase activation and amino-acid transport), argues strongly for a key role of receptor phosphorylation in insulin action.

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**Fig. 1** Dissected salivary gland complex of *H. ghilianii*. P, proboscis; AG, anterior gland; PG, posterior gland. Cells, ranging up to 1 mm or more in diameter, are clearly visible for microelectrode penetration. Scale bar, 1 mm.

## Novel preparation for studying excitation–secretion coupling in the isolated single cell

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Cellular mechanisms of secretion in exocrine and endocrine glands are technically difficult to study. We present here a model which offers fundamental advantages for studying excitation–secretion coupling at the level of the isolated single cell. The salivary gland of the leech *Haementeria ghilianii* possesses a unique combination of unusual properties which greatly facilitate research in this area. Its cells are exceptionally large (up to 1 mm in diameter), clearly visible and easy to penetrate with microelectrodes. They do not form a homogeneous population but consist of five distinct histochemical types, secreting a number of identified products such as the fibrinolytic enzyme hementin (for which there is a sensitive assay). The cells generate overshooting calcium-dependent action potentials up to 90 mV in amplitude and with a duration of 200–1,000 ms. One of their most useful and unusual features is a lack of electrical coupling which means that individual cells can be studied in an intact gland without interference from neighbouring cells.

*H. ghilianii* is the world's largest leech (up to 0.5 m long) and was known only from two nineteenth-century reports until it was rediscovered by one of us in swamps in French Guyana<sup>1</sup>. Unlike most other leeches (for example, *Hirudo medicinalis*) in which the salivary cells are relatively small and scattered throughout the head musculature<sup>2</sup>, those of *H. ghilianii* are very large and form a discrete gland containing ~240 cells (Fig. 1). Intracellular injection of Lucifer yellow indicates that the cells are not coupled because the dye does not pass into neighbouring cells<sup>3</sup>.

Leech salivary glands contain a number of medically interesting peptides, including an anti-elastase, a vasodilator, hyaluronidase, an anti-metastasis factor, a possible local anaesthetic and a species-specific range of anticoagulants<sup>4,5</sup>. For example, the anticoagulant of *H. ghilianii* is hementin, an enzyme that specifically cleaves fibrinogen/fibrin<sup>6</sup>.

These properties, together with the electrophysiological experiments reported here, suggest that the gland is a valuable model for glandular secretion, particularly because much is known about the biochemistry and mechanisms of action of some of the biologically active secretions mentioned above<sup>5</sup>. Features of the electrophysiology of this gland have recently been examined by Marshall and Lent<sup>7</sup>. Here we present the results of parallel studies and summarize the evidence which has led us to recognize the potential of this system as a gland model.

Dissected salivary glands were pinned to the base of a small Perspex bath and immersed in physiological saline<sup>8</sup>. Individual cells were impaled with two independent KCl-filled microelectrodes of 10–20 MΩ resistance; one electrode was used for recording potential and the other for passing current. Experiments were performed at room temperature (18–22 °C).

Resting potentials of between –35 and –80 mV were recorded, with no sign of spontaneous impulse activity or subthreshold potentials. Depolarizing pulses elicited overshooting action potentials which exceeded 90 mV in some cells. Action potentials were characterized by a shoulder on the repolarizing phase, pronounced after hyperpolarization (Fig. 2a, b), and a duration of 200–400 ms which increased during repetitive firing up to 1 s. In most cells only one or two action potentials could be elicited by maintained depolarization because of rapid adaptation; recovery could take many seconds or even minutes. Action potentials could, however, be elicited by termination of a hyperpolarizing pulse (Fig. 2b, c) even when depolarization produced no response. Addition of 5 mM CoCl<sub>2</sub> to the bathing medium reversibly suppressed action potential generation (Fig. 2d–f), indicating that Ca<sup>2+</sup> is the major current carrier for the action potential.

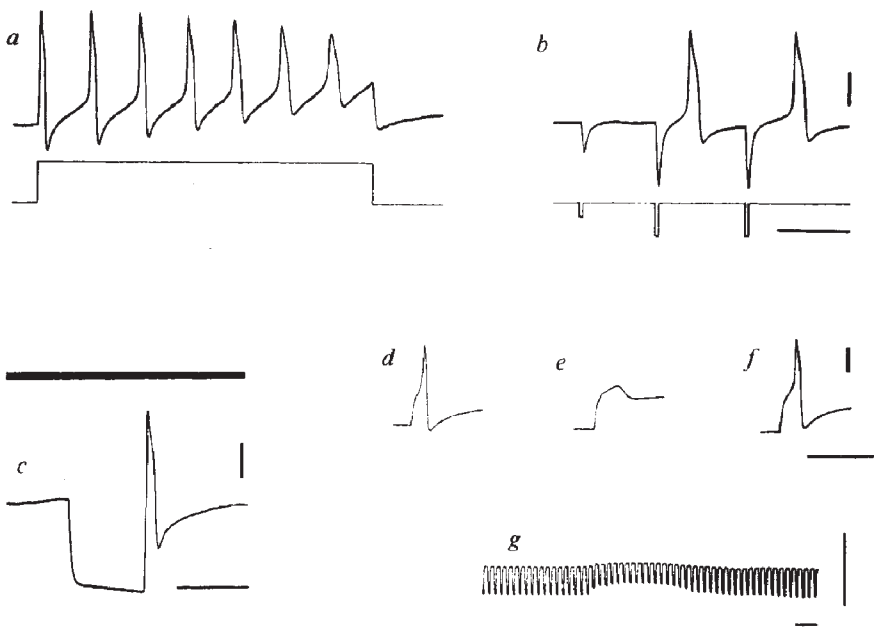
To test for electrical coupling, pairs of cells were recorded simultaneously. We never detected responses in one cell to action potentials or applied current pulses (depolarizing or hyperpolarizing) in another (Fig. 2c), even when applied pulses were increased to cause eventual damage to the cell. Both neighbouring and more distant pairs of cells were tested in this way. This lack of coupling is unusual (the only other case of electrical isolation of gland cells is in the pedal gland of molluscs<sup>9</sup>). This property should eliminate many of the experimental problems associated with coupling in other systems<sup>10</sup>. For example, it is difficult to ascertain clearly whether any electrical activity observed in a given cell originates from that or from neighbouring cells, and the effective shunt resistance of the cells may vary (for example, by the actions of transmitters on cell membrane or junctional membrane permeability).

The electrical isolation of the *H. ghilianii* salivary cells is a fundamental advantage for secretion studies at the cellular level. It should be possible, for example, to study release of identified secretions from each of the five cell types, as has been done with neurotransmitters in identified invertebrate neurones<sup>11</sup>.

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**Fig. 2** Intracellular recordings from salivary gland cells of *H. ghilianii*. *a, b*, Action potentials, which may exceed 90 mV, are initiated by depolarizing current (*a*) and also following hyperpolarization by inward current (*b*; 100-ms pulses were applied). *c*, A hyperpolarizing current pulse in one salivary cell (lower trace) produces rebound excitation but there is no sign of electrical responses in an adjacent cell, recorded simultaneously at high gain on the upper trace. Experiments of this type indicate that the gland cells are not electrically coupled. *d-f*, Effect of 5 mM  $\text{Co}^{2+}$  added to the bathing solution. *d*, Control impulse in response to depolarizing current; *e*, 5 min after addition of  $\text{CoCl}_2$  the action potential is abolished (a delayed rectification is apparent); *f*, recovery. This indicates that  $\text{Ca}^{2+}$  is the major current carrier for generation of action potentials. *g*, Brief application of  $10^{-5}$  M serotonin produces a depolarization and increase in membrane conductance (indicated by reduction in amplitude of constant-current hyperpolarizing pulses). Voltage scales (vertical bars), 25 mV (1 mV in *c*, upper trace); time scales (horizontal bars), 2 s.



The natural stimulus for action potential generation, whether neural and/or hormonal, is unknown. Several putative neurotransmitters, however, (dopamine, serotonin and acetylcholine) were found to depolarize the gland cells, with an accompanying decrease in membrane resistance (Fig. 2g) and occasionally the production of no more than four impulses. Interestingly, in the presence of dopamine, applied depolarizing current was sometimes found to produce repetitive firing which was not simply a consequence of the depolarization produced by the drug. If the impulse provides a trigger for secretion, it seems unusual that the cells are normally so difficult to activate. Feeding, however, occurs very infrequently (every few months) and an action such as that of dopamine may mimic a natural process of bringing the gland into secreting condition.

In mammals, salivary and other exocrine gland cells are electrically inexcitable, producing graded potential changes (often hyperpolarizations) which may or may not be related to secretory function<sup>10</sup>. The *H. ghilianii* salivary cells are similar in their electrical excitability to mammalian endocrine cells such as those in the pancreas<sup>12</sup>, adenohypophysis<sup>13</sup> and adrenal gland<sup>14</sup> (some molluscan exocrine glands produce action potentials<sup>15</sup>). This similarity extends to the anode-break excitation<sup>8</sup> shown by chromaffin<sup>14</sup> and anterior pituitary cells<sup>13</sup>.

We have also found the *H. ghilianii* salivary gland to be suitable for molecular genetics because the cells have a very large ramifying nucleus that displays gene amplification of  $\sim 10^6$  times; this should allow precise questions to be asked about the relationship between secretion and transcription/translation of identified genes. Thus, the *H. ghilianii* salivary gland, with its unusual combination of properties, represents a simple, accessible preparation with distinct experimental advantages for cellular studies of glandular secretion.

This specialized leech has been generally unavailable because in its natural habitat it is restricted to Amazonia. We have developed techniques for breeding this species and a facility has been set up by Biopharm to supply hementin to interested researchers.

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## Production of transgenic rabbits, sheep and pigs by microinjection

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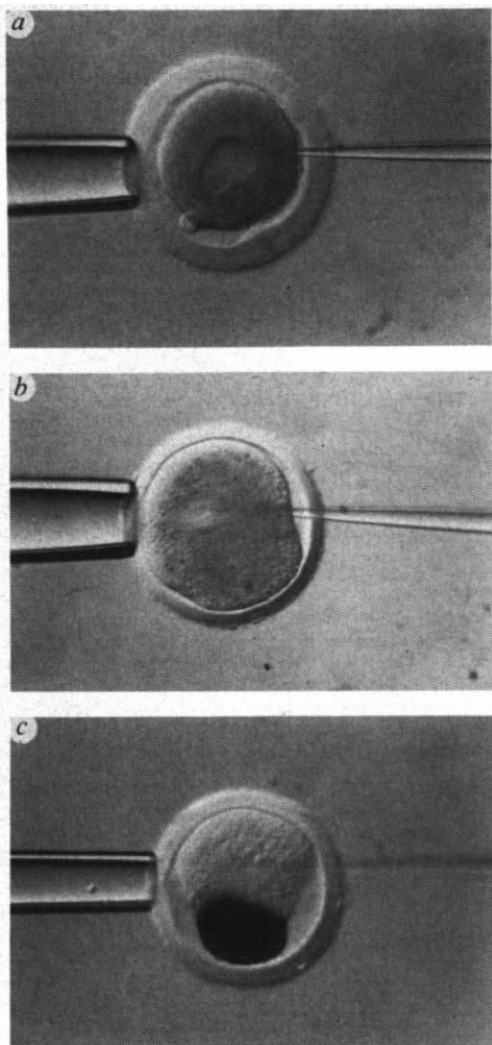
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Direct microinjection has been used to introduce foreign DNA into a number of terminally differentiated cell types as well as embryos of several species including sea urchin<sup>1</sup>, *Candida elegans*<sup>2</sup>, *Xenopus*<sup>3</sup>, *Drosophila*<sup>4,5</sup> and mice<sup>6-11</sup>. Various genes have been successfully introduced into mice including constructs consisting of the mouse metallothionein-I (MT) promoter/regulator region fused to either the rat or human growth hormone (hGH) structural genes. Transgenic mice harbouring such genes commonly exhibit high, metal-inducible levels of the fusion messenger RNA in several organs, substantial quantities of the foreign growth hormone in serum and enhanced growth<sup>12,13</sup>. In addition, the gene is stably incorporated into the germ line, making the phenotype heritable. Because of the scientific importance and potential economic value of transgenic livestock containing foreign genes, we initiated studies on large animals by microinjecting the fusion gene, MT-hGH<sup>13</sup>, into the pronuclei or nuclei of eggs from superovulated rabbits, sheep and pigs. We report here integration of the gene in all three species and expression of the gene in transgenic rabbits and pigs.

Studies with mouse ova indicated that integration of a gene into host chromosomes is much more efficient with nuclear than with cytoplasmic injection<sup>14</sup>. On this basis, we reasoned that nuclear injection would be an appropriate first approach with other species. The first problem encountered was visualization



**Fig. 1** Interference-contrast photomicrographs of one-cell fertilized ova from rabbit (*a*), sheep (*b*) and pig (*c*) following microinjection. Ova are held by a blunt holding pipette (diameter  $\sim 50 \mu\text{m}$ ); an injecting pipette (diameter  $\sim 1.5 \mu\text{m}$ ) has penetrated the zona pellucida, plasma membrane and pronuclear envelope. The tip is seen within the nucleoplasm immediately following injection of buffer containing DNA. The porcine ova (*c*) has been centrifuged at  $15,000g$  for 3 min to reveal the normally obscure pronuclei<sup>15</sup>. Visualization of nuclear structures is aided by the use of interference-contrast optics, and microinjection is carried out under  $\times 250$  magnification using a Leitz microinjecting apparatus<sup>10,14</sup>. Injection was monitored by observing the diameter of the pronucleus or nucleus, which was expanded  $\sim 50\%$ .

of the pronuclei or nuclei in the ova. Rabbit nuclear structures are readily seen (Fig. 1*a*). However, pronuclei and nuclei in sheep ova are difficult to locate and can only be seen by fluorescent microscopy using DNA specific fluorochromes (Hoechst, 33258) or by interference contrast (IC) microscopy (Fig. 1*b*). The combination of stain and ultraviolet light is damaging to the ovum (data not shown), so we used IC microscopy for microinjection. Fluorescent analysis indicated that IC microscopy is an effective method for pronuclear localization in approximately 80% of fertilized sheep eggs. Pig ova are opaque and no nuclear structures can be seen even with IC microscopy, but we found that centrifugation of pig ova at  $15,000g$  for 3 min stratifies the cytoplasm (Fig. 1*c*), leaving the pronuclei or nuclei visible<sup>15</sup>.

Once the nuclei could be visualized, microinjection was performed as described previously<sup>10,14</sup>. A few hundred copies of a 2.6 kilobase (kb) linear fragment containing the *MT-hGH* gene (see Fig. 2) were injected. Approximately 5,000 ova were injected and subsequently transferred to foster animals; about 500 of these resulted in fetuses or neonates (Table 1). The frequency

of *MT-hGH* integration was similar in the rabbit (12.8%) and the pig (11.0%) and low in sheep (1.3%). These integration efficiencies are probably accurate for the techniques being used because they are based on a large number of animals. The reasons for the lower integration frequency in these species compared to the mouse where it is  $\sim 27\%$  are unknown but could be related to factors such as the concentration of DNA, buffer composition, age of the ovum and the structure of the chromosomes<sup>14</sup>.

The number of copies of the *MT-hGH* gene that integrated was estimated by quantitative dot hybridization. Figure 2*d* shows the quantitation method as applied to transgenic pigs. Gene copy numbers ranged from 1 to 490 copies per cell (Table 2). The DNA from some of the transgenic animals was also analysed by restriction enzyme digestion of the chromosomal DNA followed by agarose gel electrophoresis and Southern blotting. Figure 2*a* shows the results obtained when the DNA was restricted with *EcoRI*, an enzyme that cuts once within the injected DNA. The probe detects two prominent bands in several rabbits and pigs. One band is close to the length of the injected DNA fragment (2.6 kb) and probably represents a tandem, head-to-tail array of the *MT-hGH* genes as is typically observed in transgenic mice<sup>10,12</sup>. The other band is approximately twice that length and might represent a head-to-head dimer, but further analysis will be required to test that possibility. When the DNA was restricted with *SstI* (Fig. 2*b*), an enzyme that cuts twice within the injected DNA, two bands of the expected size were observed in all of the pigs and rabbits. Analysis of the sheep sample with *EcoRI* (not shown) and *SstI* (Fig. 2*c*) revealed bands that were inconsistent with an intact *MT-hGH* gene, suggesting that the DNA had been trimmed or rearranged prior to integration.

Expression of the integrated genes was examined by quantitating *MT-hGH* mRNA by solution hybridization (Table 2). Only 4 of 16 rabbits analysed had any detectable *MT-hGH* mRNA in the liver, but the level was substantial in one of these. In mice, the frequency of expression of this gene is close to 70% (ref. 13). In pigs, mRNA levels were measured only in tail or ear samples because we did not want to risk adverse consequences of liver biopsy. Although tail and ear tissues are not primary sites of *MT* gene expression, we detected low levels of *MT-hGH* mRNA in several of the transgenic pigs (Table 2).

Plasma samples taken from pigs at birth and  $\sim 1$  month later were analysed for hGH by radioimmunoassay. At birth, 11 of 18 pigs had detectable levels of hGH, ranging between 2 and  $730 \text{ ng ml}^{-1}$  (Table 2). One month later, hGH exceeded  $300 \text{ ng ml}^{-1}$  in three pigs. One rabbit also had a high level of hGH. Serum hGH as high as  $64,000 \text{ ng ml}^{-1}$  has been detected in transgenic mice, but accelerated growth rate was observed at levels of 20 to  $80 \text{ ng ml}^{-1}$  (ref. 13). None of these animals were exposed to high levels of zinc, a treatment that has been shown to activate *MT-hGH* gene expression  $\sim 10$ -fold in mice<sup>13</sup>.

The effects of hGH on the growth of rabbits cannot be evaluated at present because only one live rabbit had detectable serum hGH and unfortunately it had malocclusion that impaired normal food consumption. Early indications are that the levels of hGH found in these transgenic pigs do not increase body weight dramatically. This may not be surprising considering that daily injections of bacterially synthesized hGH had no effect<sup>16</sup>, and exogenous, highly purified porcine GH only stimulated growth by 10% when delivered during the major growth phase of the pig<sup>17</sup>. Transgenic offspring and littermate controls will need to be raised on normal and zinc-supplemented diets to determine precisely the effects of hGH on growth rate and other nutritional as well as endocrine parameters.

These experiments demonstrate that foreign genes can be introduced into several large animal species by microinjection of ova. Furthermore, expression of *MT-hGH* was obtained in rabbits and pigs. We used a fusion gene that has worked well in mice to demonstrate the feasibility of such techniques, and we are now trying several modifications in an effort to improve the level of expression and physiological response.



**Table 1** Efficiency of producing *MT-hGH* transgenic rabbits, sheep and pigs by microinjection

| Species | Transferred injected ova | Recipients | Integration frequency (%) | Expression frequency <i>MT-hGH</i> mRNA | Serum or plasma hGH |
|---------|--------------------------|------------|---------------------------|-----------------------------------------|---------------------|
| Rabbit* | 1,907                    | 73         | 28/218 (12.8)             | 4/16                                    | 1/1                 |
| Sheep†  | 1,032                    | 192        | 1/73 (1.3)                | ND                                      | ND                  |
| Pig‡    | 2,035                    | 64         | 20/192 (10.4)             | 11/20                                   | 11/18               |

Integration frequency is the number of animals (fetuses, stillborns and neonates) that retained the injected DNA/total number of animals resulting from injected ova. Six gilts bearing only injected eggs farrowed, producing 52 neonates, 5 of which retained DNA. In 31 gilts that farrowed, 204 fertilized control ova were transferred along with 859 injected ova to ensure sufficient embryos at implantation to maintain pregnancy. If survival of injected eggs to fetuses (16.4%) was similar for both groups, then injected eggs resulted in 140 of 252 fetuses and piglets produced, 15 of which retained injected DNA. We combined the data from the two groups to estimate integration efficiency. Expression frequency is the number of fetuses or neonates containing *MT-hGH* mRNA or plasma hGH per total number of animals examined. ND, not determined.

\* Fertilized one-cell rabbit ova were flushed from the oviducts of superovulated New Zealand White (NZW) females 19 h after mating<sup>20</sup>. For microinjection the ova were placed in the well of a depression slide containing ~100 µl modified BMOC culture medium<sup>21</sup> with the NaHCO<sub>3</sub> replaced by 25 mM HEPES<sup>22</sup> and covered by silicone oil. Microinjection of embryos (1,857 one-cell and 50 two-cell) was performed as described for mouse ova<sup>10,14</sup>. Following injection, the ova were washed in fresh modified BMOC and surgically transferred to the oviducts of synchronized pseudopregnant rabbits<sup>23</sup>.

† Rambouillet ewes were superovulated after exhibiting at least one prior oestrus period. On about day 10 of the oestrous cycle, progestagen-impregnated vaginal sponges (6α-methyl-17α-acetoxy progesterone, 60 mg; from Dr J. Lauderdale, Upjohn) were inserted and left for 12 days. Gonadotropin treatment (porcine follicle stimulating hormone, Burns) began three days before sponge removal and was continued twice daily (2.5 mg per injection, intramuscular) until the day following sponge removal<sup>24</sup>. At the onset of oestrus, ewes were either hand mated to fertile rams or inseminated *in utero* with 0.2 ml per horn of washed ram semen; 72 h after sponge removal, one-cell fertilized ova and cleaved ova were surgically collected from the reproductive tracts of anaesthetized ewes by flushing 6 ml Ham's F-10 medium containing 10% heat-inactivated fetal calf serum (FCS) from the utero-tubal junction through the cannulated infundibular end of each oviduct. The flushings were collected in sterile Petri dishes, and ova were removed under a dissecting scope. Ova were transferred to fresh Ham's F-10 containing 10% FCS and transported (~2.5 h) to Philadelphia in temperature-controlled containers. Microinjection of embryos (641 one-cell, 375 two-cell and 16 four-cell) was performed as previously described<sup>10,14</sup>. After embryos were injected, they were washed and transported to Beltsville. Embryos were aspirated into a glass micropipet tip with 10 µl Ham's F-10 and expelled 1–3 cm into the fimbriated end of the oviduct in synchronized recipient ewes. To assess the effects of transport and microinjection of DNA on egg development, a number of recipients bearing control and injected eggs were flushed 8 days following transfer. In recipients in which eggs were recovered, 26% of transported, uninjected and 10% of injected sheep eggs developed to blastocysts. Because of the high mortality of transported, injected eggs and the concern of multiple births, 5 or 6 embryos were transferred per recipient.

‡ Mature gilts were superovulated and bred as previously described<sup>15</sup>. At 18 to 27 h after the expected time of ovulation, gilts were anaesthetized and one cell fertilized the oviduct with 20 ml modified BMOC<sup>21</sup>. Ova were transferred to fresh BMOC and transported to Philadelphia. Microinjection of embryos (316 one-cell, and 1,719 two-cell) was performed as previously described<sup>10,14</sup>. The obscured pronuclei or nuclei of one- and two-cell pig ova were visible after centrifugation for 3 min at 15,000 g. Centrifugation of pig ova at this force and length of time has no detectable effect on development<sup>15</sup>. After embryos were injected, they were transported to Beltsville and transferred to the oviducts of recipient gilts as previously described<sup>15</sup>. To assess the effects of transport and microinjection of DNA on egg development, recipients bearing control and injected eggs were flushed 5 days following transfer. Approximately 52% of transported, uninjected and 23% of injected pig eggs developed to blastocysts. The pregnancy rate in recipients bearing only injected eggs was 50% while in recipients bearing both injected and control eggs 58% farrowed.

**Table 2** Characteristics of transgenic rabbits, sheep and pigs

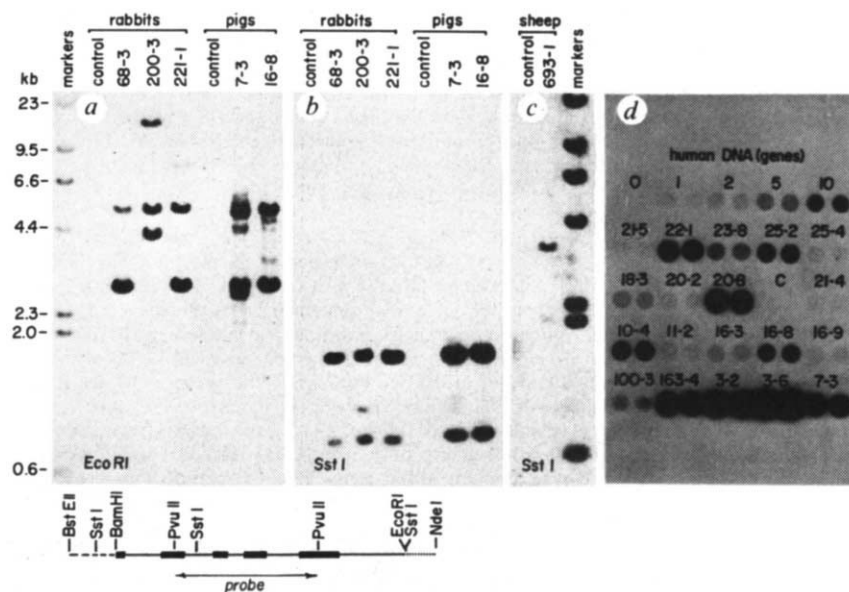
| Species | Animal and sex | Gene copy (no. per cell) | <i>hGH</i> mRNA (molecules cell <sup>-1</sup> ) | Immuno-assayable <i>hGH</i> (ng ml <sup>-1</sup> ) | Species | Animal and sex | Gene copy (no. per cell) | <i>hGH</i> mRNA (molecules cell <sup>-1</sup> ) | Immuno-assayable <i>hGH</i> (ng ml <sup>-1</sup> ) |
|---------|----------------|--------------------------|-------------------------------------------------|----------------------------------------------------|---------|----------------|--------------------------|-------------------------------------------------|----------------------------------------------------|
| Rabbit  | 59-3*          | 20                       | 0                                               |                                                    | Pig     | 100-3*         | 4                        | 0                                               | ND                                                 |
|         | 64-2♀          | 18                       | 0                                               |                                                    |         | 163-4*         | 140                      | 0                                               | ND                                                 |
|         | 68-3†          | 28                       | 39                                              | ND                                                 |         | 3-2♂           | 330                      | 26                                              | Neg.                                               |
|         | 68-4♀          | 24                       | 0                                               |                                                    |         | 3-6♂           | 490                      | 53                                              | 17                                                 |
|         | 122-9♀         | 11                       | 0                                               |                                                    |         | 7-3♀           | 90                       | 12                                              | 80                                                 |
|         | 131-8†         | 88                       | 0                                               |                                                    |         | 10-4♀          | 23                       | 0                                               | Neg.                                               |
|         | 157-1♀         | 3                        | 0                                               |                                                    |         | 11-2♂          | 1                        | 0                                               | 40                                                 |
|         | 163-3♂         | 3                        | 15                                              | 250                                                |         | 16-3♀          | 3                        | 0                                               | Neg.                                               |
|         | 167-5♀         | 10                       | 0                                               |                                                    |         | 16-8♀          | 10                       | 18                                              | 53                                                 |
|         | 179-1♀         | 16                       | 0                                               |                                                    |         | 16-9♂          | 1                        | 5                                               | 65                                                 |
|         | 179-2♂         | 36                       | 0                                               |                                                    |         | 17-4♀          | 3                        | 0                                               | Neg.                                               |
|         | 179-5♀         | 6                        | 0                                               |                                                    |         | 18-3♀          | 3                        | 6                                               | 60                                                 |
|         | 200-3*         | 8                        | 920                                             | ND                                                 |         | 20-2♂          | 2                        | 4                                               | 40                                                 |
|         | 221-1*         | 40                       | 140                                             | ND                                                 |         | 20-8♂          | 110                      | 1                                               | 2                                                  |
|         | 223-4*         | 5                        | 0                                               |                                                    |         | 21-4♂          | 1                        | 2                                               | Neg.                                               |
|         | 223-5*         | 40                       | 0                                               |                                                    |         | 21-5♀          | 1                        | 0                                               | Neg.                                               |
| Sheep   | 693-1♀         | 1                        | ND                                              | ND                                                 |         | 22-1♀          | 50                       | 24                                              | 56                                                 |
|         |                |                          |                                                 |                                                    |         | 23-8♀          | 7                        | 41                                              | 730                                                |
|         |                |                          |                                                 |                                                    |         | 25-2♀          | 17                       | 0                                               | 108                                                |
|         |                |                          |                                                 |                                                    |         | 25-4♀          | 2                        | 0                                               | Neg                                                |
|         |                |                          |                                                 |                                                    |         |                |                          |                                                 |                                                    |

A 2.6-kb linear fragment of the fusion gene *MT-hGH*<sup>13</sup> containing the mouse MT-I regulator/promoter fused to *hGH*<sup>19</sup> was injected into fertilized one-cell and two-cell rabbit, sheep and pig eggs as described for mouse ova<sup>10,14</sup>. The male or female pronuclei of one-cell ova and both nuclei of two-cell ova were microinjected with a 3 ng µl<sup>-1</sup> solution of DNA in Tris-EDTA buffer<sup>14</sup>. The ova were transferred into the oviducts of recipients at the same stage post oestrus as the donors (see Table 1). Animals without identified gender were either killed as fetuses(\*) or were stillborn(†). The number of foreign fusion genes per cell was estimated by extracting total nucleic acids from a piece of fetal liver, neonatal ear or tail samples and performing quantitative dot hybridization with a 1.0-kb *Pvu*II probe spanning most of the *hGH* structural gene<sup>19</sup> (see Fig. 2). *MT-hGH* mRNA was measured by solution hybridization with a <sup>32</sup>P-labelled oligonucleotide (21-mer)<sup>25</sup>. For rabbits, either a partial hepatectomy was performed or fetal liver was used. For pigs, the *MT-hGH* mRNA content of ear or tail samples was quantitated. The concentration of *hGH* was measured in pig plasma obtained shortly after birth and serum from a 9-month-old rabbit. Samples were assayed in duplicate at 2.5 and 10 µl by radioimmunoassay using a *hGH* kit provided by Dr Raiti (National Hormone and Pituitary Program). The assay did not cross-react with porcine GH but required extra normal rabbit serum and anti-rabbit gammaglobulin to quantify *hGH* in rabbit samples. Pigs with *hGH* values less than 2 ng ml<sup>-1</sup> at birth were designated negative for *hGH*. At about one month of age, these pigs were also negative for *hGH*. ND, not determined; Neg, negative for *hGH*.

The key element in our success was the ability to visualize pronuclei and nuclei. Microinjection of the *MT-hGH* gene into the cytoplasm of 485 pig ova failed to produce DNA integration in 42 fetuses. Separate techniques for sheep and pigs were

necessary because the opacity of the eggs differed. Although both contain dense cytoplasm, centrifugation did not help visualize pronuclei of sheep eggs and IC microscopy did not allow nuclear localization in pig ova. Preliminary work indicates

**Fig. 2** Analysis of *MT-hGH* DNA introduced into rabbits, pigs and sheep. The diagram at the bottom shows the 2.6-kb *Bst*EII/*Eco*RI DNA fragment isolated from *MT-hGH* gene plasmid 111 that was microinjected<sup>13</sup>; the mouse *MT-I* promoter region is dashed, the *hGH* gene is solid, with the exons indicated as boxes, and the residual pBR322 sequences are dotted. The internal *Pvu*II fragment was isolated, nick-translated and used as a probe for quantitation of genes and Southern blots. Panels *a*, *b* and *c*: DNA (5 µg for controls and transgenic animals 200-3, 16-8 and 693-1; 1 µg mixed with 4 µg of control DNA for 68-3, 221-1 and 7-3) were digested with the indicated restriction enzymes (10 units, 6 h), electrophoresed on a 1% agarose gel, transferred to nitrocellulose and hybridized with the nick-translated probe, washed and autoradiographed as described previously<sup>18</sup>. *a* and *b*, Exposure was 4.5 h; *c*, 24 h. For quantitation of gene copy number, 5 µg of DNA was spotted in duplicate onto nitrocellulose along with standards of 0, 0.5, 1, 2 and 5 µg of human DNA mixed with control DNA to make a total of 5 µg. *d* Shows the visualization *MT-hGH* gene copy number in transgenic pigs. After exposure, the spots were cut out and the radioactivity determined in a scintillation counter. Gene copy numbers were calculated from the standards assuming that the genome size of pigs and humans are comparable and that diploid human cells contain 10 genes homologous to the *Pvu*II fragment used as probe<sup>19</sup>. The results are shown in Table 2.



that these two techniques can be used for ova of other species; for example, IC microscopy allowed visualization of pronuclei in goat ova (unpublished observations) and centrifugation allowed localization of pronuclei in cow ova<sup>15</sup>. Although improvements in integration efficiency should be possible, the techniques have immediate application for both scientific and practical purposes.

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## Expression of active human clotting factor IX from recombinant DNA clones in mammalian cells

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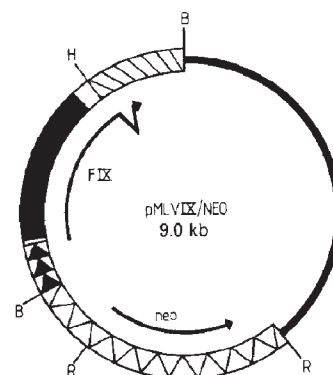
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Haemophilia B, or Christmas disease, is an inherited X-chromosome-linked bleeding disorder caused by a defect in clotting factor IX and occurs in about 1 in 30,000 males in the United Kingdom<sup>1</sup>. Injection of factor IX concentrate obtained from blood donors allows most patients to be successfully managed. However, because of impurities in the factor IX concentrate presently in use, this treatment involves some risk of infection by blood-borne viruses such as non-A, non-B hepatitis and the virus causing acquired immune deficiency syndrome (AIDS)<sup>2</sup>. Because of the recent concern about the increasing incidence of AIDS amongst haemophiliacs, a factor IX preparation derived from a source other than blood is desirable. Here, we report that after introduction of human factor IX DNA clones<sup>3</sup> into a rat hepatoma cell line using recombinant DNA methods, we were able to isolate small amounts of biologically active human factor IX.

Factor IX is a plasma glycoprotein which has an essential role in the middle phase of the intrinsic clotting pathway<sup>4</sup> where, in an activated form, IXa, it interacts with factor VIII, phospholipid and calcium ions to form a complex that converts factor X to Xa. Factor IX is synthesized in liver hepatocytes where it undergoes three distinct types of post-translational modification before secretion into the bloodstream as a 415-amino-acid-long, highly modified protein. These modifications are the vitamin K-dependent  $\gamma$ -carboxylation of 12 glutamic acid residues<sup>5</sup>, the addition of several carbohydrate residues<sup>6</sup> and the  $\beta$ -hydroxylation of a single aspartic acid residue<sup>7</sup>. The first two modifications are known to be required for activity of factor IX<sup>5,6</sup>. Because of the complex and specialized nature of these modifications, it seemed probable that the expression of active factor IX, derived from factor IX DNA clones, would be most likely to succeed in a hepatic cell or a transformed cell line derived from a hepatocyte. None of the standard mammalian hepatoma cell



**Fig. 1** Map of the expression plasmid pMLVIX/NEO. Restriction sites for *Eco*RI, *Bam*HI and *Hind*III are denoted R, B and H, respectively. The factor IX (abbreviated FIX) and *tk/neo* (abbreviated neo) sections are indicated by arrows which show the 5'→3' direction of the mRNA transcribed from these genes. pAT153 sequences are indicated by the narrow solid line, the *tk/neo* gene by the zig-zag line. The factor IX transcription unit, from 5' to 3', consists of the M-MuLV LTR (▲), factor IX 5'-noncoding sequence (open wide line), factor IX coding sequence (solid wide line), factor IX 3'-noncoding sequence (open wide line) and SV40 sequences (diagonals).



**Methods.** Construction of the plasmid: The region from nucleotides 25 to 93 was removed from the factor IX cDNA cVI by digesting cVI<sup>3</sup> (held in the plasmid vector pAT153/PvuII/8) with *Bam*HI, followed by 'filling-in' the overhanging ends with deoxynucleoside triphosphates using the Klenow fragment of DNA polymerase I (*Escherichia coli*). After dephosphorylation with calf intestinal phosphatase, a partial digest with *Eco*RV was performed. *Eco*RV recognizes the sequence GATATC at nucleotides 91–96. A partial digest was necessary because of the presence of an additional *Eco*RV site at nucleotide 508. The resulting linear fragment of ~4.9 kb was purified by agarose gel electrophoresis. A short (0.1-kb) *Taq*I/*Eco*RV fragment was generated from the factor IX genomic DNA XI<sup>3</sup> by digesting XI (also held in the plasmid vector pAT153/PvuII/8) with *Taq*I, filling-in the overhanging ends with deoxynucleoside triphosphates as before in the presence of tracer amounts of [ $\alpha$ -<sup>32</sup>P]dGTP and re-cutting with *Eco*RV. This labelled fragment, corresponding to nucleotides 288–386 of the genomic DNA (see Fig. 4 of ref. 3), was separated from other *Taq*I and *Eco*RV fragments derived from the plasmid vector by 6% acrylamide gel electrophoresis. The 4.9-kb and 0.1-kb fragments were ligated together and cloned in *E. coli* MC1061. One clone, designated p5'G3'cVI, contained the reconstructed factor IX gene corresponding to nucleotides 1–1,572 of the mRNA<sup>3</sup>, effectively adding nucleotides 1–25 to, and correcting the rearrangement in, the first 15 residues of clone cVI. The clone also contained eight extra nucleotides, genomic DNA residues 288–295, derived from the presumed factor IX promoter region. The factor IX DNA insert present in p5'G3'cVI was removed by restriction with *Bam*HI and *Hind*III and isolated by agarose gel electrophoresis. Construction of the expression vector pMLV/NEO: pTKMoLTR1 (given by Dr J. C. Lang<sup>20</sup>) containing the M-MuLV LTR and the herpes simplex virus *tk* gene, was restricted with *Hind*III and *Bam*HI to remove the *tk* coding sequence. The remainder of the molecule (pAT153 and M-MuLV LTR) was purified by agarose gel electrophoresis, then used as a vector for cloning of an agarose gel-purified 0.9-kb *Bam*HI/*Hind*III fragment containing the SV40 small-t intron and early gene polyadenylation signal which was derived from pSV1<sup>17</sup> (given by Dr Colman). Recombinants containing the 0.9-kb fragment were identified by restriction mapping and one was chosen and designated pMLV. pMLV was restricted with *Pvu*I and *Eco*RI, and the *Eco*RI ends blunt-ended by filling in using the Klenow fragment of DNA polymerase I. The 4.4-kb *Pvu*I/*Eco*RI fragment containing the M-MuLV LTR, SV40 sequences and most of the pAT153 sequences, was purified by agarose gel electrophoresis. The *tk/neo* gene and part of the ampicillin resistance gene (*amp*<sup>r</sup>)<sup>9</sup> was purified as a *Pvu*I/*Bam*HI fragment from the cosmid vector pTM<sup>18</sup>. The *Bam*HI end was blunt-ended by filling in (see above). This fragment was then ligated to the pMLV-derived *Pvu*I/*Eco*RI fragment using T4 DNA ligase and recombinants selected on ampicillin. Note that the *Pvu*I site interrupts the *amp*<sup>r</sup> gene so that in this ligation the *amp*<sup>r</sup> gene was reconstructed in part from one fragment and in part from the other. The structure of recombinants was checked by restriction mapping and one clone, designated pMLV/NEO, was used in the next stage. The isolated factor IX DNA from stage 1 (above) was ligated into the *Hind*III site of the expression vector pMLV/NEO using T4 DNA ligase and the Klenow fragment of DNA polymerase I. Clones containing the factor IX DNA insert in the correct orientation were identified by restriction mapping, and one such clone, pMLVIX/NEO, was used for transfection.

lines secrete active endogenous factor IX. The rat hepatoma line H4-11-E-C3, however, is known to secrete a biologically active form of another vitamin K-dependent clotting factor, prothrombin<sup>8</sup>, indicating the presence of an active  $\gamma$ -carboxylase system; therefore we chose this cell line for our studies.

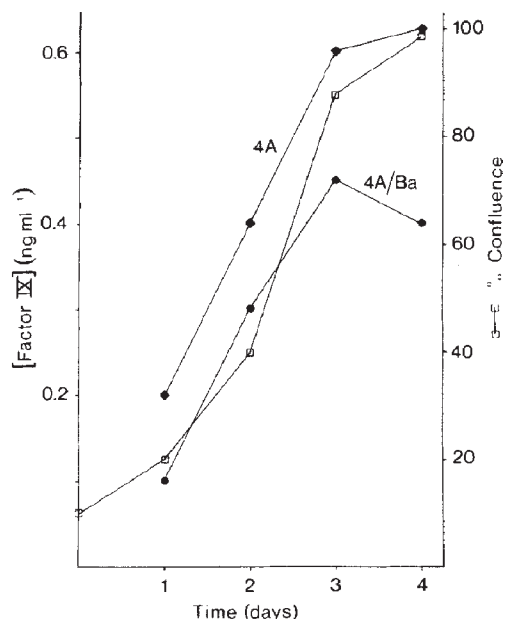
We first constructed a factor IX expression plasmid (Fig. 1) from previously isolated human complementary DNA and genomic clones as follows. A full-length factor IX cDNA clone, p5'G3'cVI, was assembled from the cDNA clone cVI<sup>3</sup> and a small fragment of genomic factor IX sequence encoding the 5'-noncoding sequence of the normal mRNA and the first domain of the protein. The resulting cDNA sequence was then placed under the transcriptional control of the promoter of the long terminal repeat (LTR) of Moloney murine leukaemia virus (M-MuLV). The transcription unit was completed by placing the simian virus 40 (SV40) small-t-antigen intron and early polyadenylation signal downstream of the factor IX coding sequence. The plasmid also contains the *tk/neo* gene<sup>9</sup> in order to provide a dominant selectable marker for expression in mammalian cells. In preliminary experiments, we tested the expression plasmid pMLVIX/NEO, by transfecting<sup>10</sup> mouse fibroblast Ltk<sup>-</sup> cells and selecting permanent cell lines for resistance to the antibiotic G418 (ref. 11). One cell line was characterized in detail and secreted detectable human factor IX in an enzyme-linked immunosorbent assay, but the secreted factor IX failed to adsorb to barium sulphate (see Fig. 2 legend and ref. 12 for assay details). Thus, the expression plasmid was proved functional in producing a protein. However, as expected, the mouse fibroblast cell line lacked the enzymes required for  $\gamma$ -carboxylation of the N-terminal glutamyl residues of factor IX, which are necessary for its biological activity.

The rat hepatoma line H4-11-E-C3 (see above) was transfected with the factor IX expression plasmid by standard calcium phosphate precipitation methodology<sup>10</sup>. Cells containing a functional *tk/neo* gene were selected for G418 resistance<sup>11</sup> and 24

of the resultant colonies were cloned by limiting dilution. These permanent cell lines were then grown up and the growth medium of each assayed for the presence of secreted human factor IX by ELISA (see Fig. 2 legend). Four of the cell lines secreted detectable levels of factor IX and the highest producer, line 4A, was chosen for further analysis. Southern blot analysis indicated that there was only one copy of the plasmid integrated into the chromosomal DNA per cell (data not shown). Northern blot analysis of total cellular RNA derived from line 4A revealed the presence of a factor IX mRNA species of ~1.6 kilobase (kb), which is the expected size of factor IX mRNA. On both Southern and Northern blot analysis, no cross-hybridizing species were detected in the control hepatic cell line H4-11-E-C3 (data not shown).

Figure 2 shows the time course of secretion of human factor IX by line 4A as measured by a specific, quantitative ELISA (see Fig. 2 legend); from this experiment, we estimate the rate of secretion of factor IX to be ~6 ng per 10<sup>7</sup> cells per 24 h. Furthermore (see Fig. 2), adsorption of the secreted factor IX onto barium sulphate<sup>12</sup>, a procedure which depends on the  $\gamma$ -carboxylation of factor IX, suggests that the protein is  $\gamma$ -carboxylated. As the recovery of factor IX was not corrected for any losses during the experimental procedure, we cannot distinguish between the possibility that either about 70% of the material has  $\gamma$ -carboxyglutamic acid residues or, alternatively, that the material is all  $\gamma$ -carboxylated and there were losses of ~30% during recovery of the protein. No factor IX was detectable in lysates of the 4A cells, indicating that there was insignificant accumulation within the cell. No cross-reacting material was detected either in control H4-11-E-C3 conditioned media or in cell lysates.

Table 1 shows the results of a clotting and antigenic analysis of a sample of affinity-purified human factor IX from conditioned medium of line 4A cells, together with an analysis of a parallel control sample of H4-11-E-C3 cells. The purified human



**Fig. 2** Secretion of human factor IX by clone 4A cells. ◆, Factor IX levels; ●, factor IX recoverable by barium sulphate adsorption; □, degree of confluency.

**Methods.** Cells were seeded at 10% confluency in an 80-cm<sup>2</sup> flask containing 10 ml of minimal essential medium (MEM: 10% fetal calf serum containing penicillin, streptomycin, kanamycin and 100 ng ml<sup>-1</sup> vitamin K). The medium was changed every 24 h and the conditioned medium assayed for human factor IX by ELISA. The ELISA was done by first binding 0.2 ml of a mouse anti-factor IX monoclonal antibody 3A6<sup>14</sup>, diluted 1:5,000 in 200 mM sodium carbonate pH 9.0, to microtitre wells at room temperature for 2 h. Samples (150 µl) of conditioned medium were then bound overnight at 4°C. The second antibody, 150 µl of rabbit anti-human factor IX (Calbiochem-Behring), diluted 1:500 in PBS, 1% NP40 and 10% horse serum, was then bound at room temperature for 2 h. The third antibody, peroxidase-conjugated swine anti-rabbit IgG (Dako), was bound in the same manner. The assay was finally developed with 0.2 ml of 40 mM ABTS (azinodi-(3-ethylbenzothiazole sulphonic acid)-diammonium salt) in 0.008% H<sub>2</sub>O<sub>2</sub> in 50 mM citrate buffer pH 4.0 for 30–45 min. Colour change of the solution was measured at 405 nm in a spectrophotometer. A standard curve was constructed using twofold dilutions of normal plasma in the range 10<sup>-3</sup>–10<sup>-5</sup> dilution, assuming a concentration of 5 µg ml<sup>-1</sup> (ref. 1) for plasma factor IX. Undetectable quantities (<0.02 ng ml<sup>-1</sup>) of factor IX were observed in conditioned medium from control H4-11-E-C3 cells using ELISA.

factor IX is active in a one-stage clotting assay<sup>13</sup>, giving 7% of the activity of normal plasma. The control shows only 1% activity and this is presumably due to the trace amounts of bovine factor IX which were found in both line 4A and control samples, as indicated by an ELISA for bovine factor IX antigen (see Table 1). Further evidence (Table 1) that the cell line 4A is secreting active human factor IX is provided by the inhibitory effect of the specific anti-human factor IX monoclonal antibody 3A6<sup>14</sup> in the clotting assay. Control experiments showed that this antibody does not significantly inhibit bovine or rat factor IX activity at the concentration used here (data not shown). The fact that the activity level (7% of normal plasma) in the line 4A sample correlates well with the level of human factor IX antigen (6.8% of normal plasma) suggests that the protein is fully active. We conclude that line 4A cells secrete biologically active human factor IX, albeit at a low level.

We are testing alternative expression plasmids and attempting to isolate improved cell lines which might secrete more factor IX, thus facilitating purification of larger amounts of material. This should allow a detailed examination of the properties of the genetically engineered factor IX protein in comparison with the plasma protein, and should eventually allow us to start *in vivo* experiments of the kind necessary for the development and testing of a therapeutic product. For example, it is important to determine the half-life and efficacy of the genetically

**Table 1** Analysis of clotting factor IX activity and of human and bovine factor IX antigen in purified samples from 4A and H4-11-E-C3 conditioned media

| Assay                       | Cell line 4A | H4-11-E-C3 |
|-----------------------------|--------------|------------|
| Clotting (%)                | 7            | 1          |
| Clotting (%) + 3A6 antibody | <1           | <1         |
| Human IX antigen (%)        | 6.8          | ND         |
| Bovine IX antigen           | 0.8          | 1.2        |

Factor IX clotting activity (measured using the one-stage clotting assay<sup>13</sup> in which factor IX-deficient plasma is used as a substrate), and human factor IX antigen (measured by ELISA) are both given as percentages of average normal plasma levels. The inhibitory effect of the monoclonal antibody 3A6 was measured by preincubation of the sample to be tested with a final concentration of 2.5% 3A6 ascites fluid for 15 min at room temperature. Bovine antigen is given in arbitrary units and was assayed by quantitative ELISA as follows. Dilutions of pure bovine factor IX or samples to be assayed were diluted in 200 mM sodium bicarbonate, pH 9.0 buffer and dried onto microtitre plates. Bovine factor IX was then detected using a polyclonal rabbit anti-bovine factor IX antibody and a peroxidase-conjugated anti-rabbit IgG antibody as for the ELISA for human factor IX. The human factor IX sample from 1.5 l of conditioned media from line 4A cells, and the control sample from the same volume of H4-11-E-C3 cells were purified by barium citrate adsorption<sup>16</sup> and immunoaffinity chromatography. An anti-human factor IX antibody affinity column was prepared by binding the 3A6 monoclonal antibody (purified from ascites fluid by sodium sulphate precipitation) to the support Affigel 10 (Biorad) using conditions recommended by the manufacturer. The sample to be bound was passed twice over the column (vol. 0.3 ml) then washed with >100 vol. phosphate-buffered saline (PBS), 1% Nonidet P-40 (NP40) and a similar volume of PBS, 1 M NaCl, 1% NP40. Finally, the column was rinsed with 5 vol. PBS, 1 M NaCl and eluted with 7 M urea, 1 M NaCl, and 0.4-ml fractions were collected. The first three fractions were pooled and immediately dialysed overnight against 2% PBS at 4°C, followed by freeze-drying. The samples were finally dissolved in 200 µl H<sub>2</sub>O. ND, not determined.

engineered product compared with plasma factor IX. A clinical product derived from the cell line described here is therefore not an immediate prospect, but the results reported here represent an important first step in demonstrating the feasibility of this approach. However, we are optimistic that further studies in conjunction with work already done on clotting factor VIII(C)<sup>15,19</sup>, will eventually allow the majority of haemophiliacs to be treated with improved virus-free products.

We thank Professor A. Bloom and Dr Ian Peake for the gift of anti-factor IX monoclonal antibody 3A6, Dr M. P. Esnouf for the gifts of pure bovine factor IX, rabbit anti-bovine factor IX antiserum and vitamin K, and the Schering Corporation for G418. We also thank Dr Charles Rizza, Dr Ian Jones and Peter Winship for useful discussions, and Barbara Paxman for secretarial help. This work was supported by MRC project grant G8405633CB to D.S.A. and G.G.B.

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# Lattice mobility and anomalous temperature factor behaviour in cytochrome *c'*

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Atomic temperature factors (*B*-values) obtained from X-ray refinement experiments provide empirical estimates of protein mobility that have been correlated with both theoretical simulations of protein dynamics<sup>1</sup> and experimental studies of antibody reactivity<sup>2,3</sup>. The comparison of *B*-values with protein solution properties requires adjustment of the apparent atomic mobilities to compensate for the effects of the crystal environment<sup>4-6</sup>. Here we compare crystallographically independent subunits of the dimeric cytochrome *c'* from the bacterium *Rhodospirillum rubrum* to examine how lattice effects influence refined *B*-values. In addition to local effects on protein mobility at crystal contacts<sup>4,5</sup>, we show that *B*-value differences up to 12 Å<sup>2</sup> between subunits result from lattice disordering effects that approximate to concerted rotations of the molecules about a crystal symmetry axis.

Ferricytochrome *c'* from *R. rubrum* is a dimeric haem protein composed of identical 128-residue polypeptide chains. The protein crystallizes in the orthorhombic space group  $P2_12_12_1$  ( $a = 56.6$ ,  $b = 72.4$ ,  $c = 75.4$  Å), with a dimer in the crystallographic asymmetrical unit<sup>7</sup>. The structure has been crystallographically refined to an *R*-factor of 0.19 for 30,533 reflections with  $F > 2\sigma F$  to 1.67 Å resolution<sup>8</sup>. Independently refined subunits are related by a non-crystallographic dyad axis with an r.m.s. difference between corresponding  $\alpha$ -carbon positions of 0.40 Å. Many detailed features of the subunits, including the equivalent situations of some 40 bound solvent molecules per monomer, are conserved despite differing environments in the crystal lattice.

Figure 1*a,b* plots mean residue *B*-value against sequence number for the two subunits of the native dimer. Comparison of the subunits yields a correlation coefficient of 0.82. This is similar to values reported for structures determined in different crystal forms<sup>5,9</sup> and is indicative of the extent to which *B*-value behaviour may reflect intrinsic protein dynamical properties<sup>1,4</sup>. Despite the similarities, monomer *B*-values differ by up to 30 Å<sup>2</sup> locally and by some 6 Å<sup>2</sup> overall (Fig. 1*c*). These variations between chemically identical subunits are attributable to their different environments in the crystal lattice.

Lattice interactions could potentially affect *B*-value behaviour in a variety of ways<sup>6</sup>. One result of the intermolecular interactions formed at crystal contacts is the localized immobilization of protein side chains or surface backbone loops. Consequently, the *B*-values of groups involved in such interactions may imply local mobilities that are substantially smaller than actually occur in solution. Previous studies have correlated the magnitude of such localized effects with solvent-accessible surface area in the crystal lattice<sup>5,10</sup>. Comparison of the mean residue *B*-values and fractional accessible surface areas<sup>11</sup> for the individual cytochrome *c'* subunits yields correlation coefficients (0.69 and 0.61, Fig. 1*a, b*) comparable to those observed previously<sup>5,10</sup>. A difference plot between monomers (Fig. 1*c*) shows that *B*-value and accessible area variations frequently correspond to alternative lattice contacts made by the two monomers in the asymmetrical unit. Nevertheless, differences between monomers correlate relatively poorly (0.35), in part resulting from an average difference of 6 Å<sup>2</sup> in subunit *B*-value that is uncorrelated with any overall difference in accessible surface area. As shown below, these variations reflect a second type of lattice effect that involves

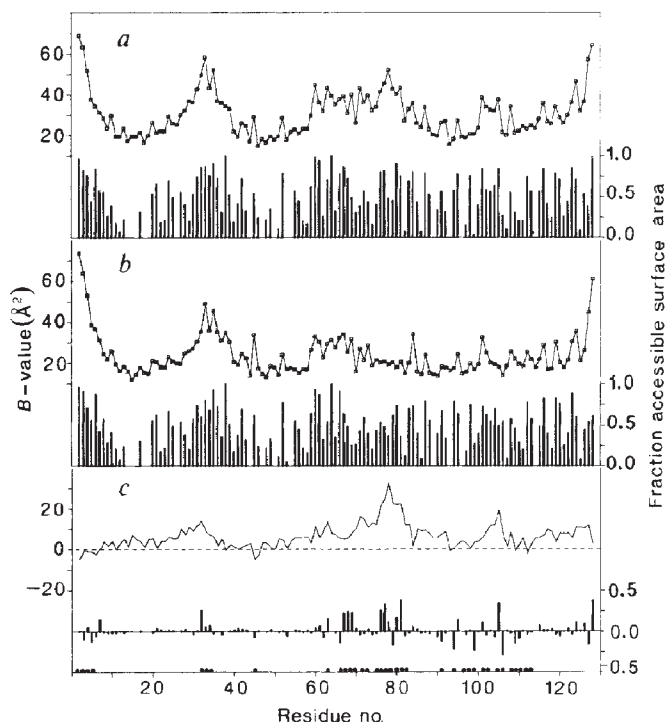


Fig. 1 *a, b*, Plots of residue *B*-value averaged over main-chain backbone atoms (connected curves), and fractional accessible surface areas<sup>5,11</sup> for entire residues in their respective crystal lattice environments (bars), against sequence number for crystallographically independent subunits of cytochrome *c'*. *c*, *B*-value (solid curve) and accessible surface area (bars) difference between subunits. Dots indicate sequence positions of residues that make different crystal contacts in the two subunits.

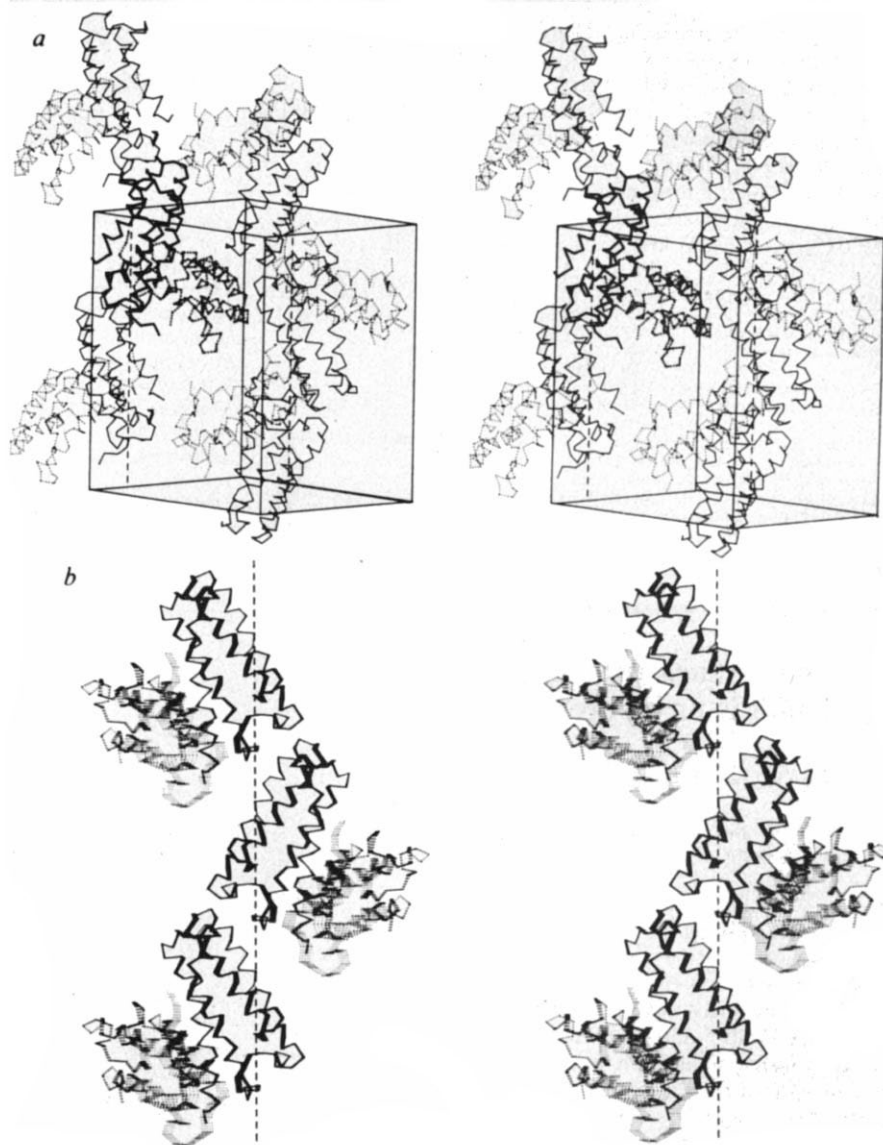
systematic disordering of molecules relative to crystal symmetry axes.

Figure 2 shows the crystal packing geometry and illustrates that subunits with systematically low *B*-values (cool monomers) form continuous lattice interactions along alternate 2-fold screw axes parallel to the unit cell *c*-axis. Hot monomers make native dimer interactions with cool monomers aligned along each crystal screw, together with lattice contacts to cool monomers aligned along adjacent *c*-axis screws (Fig. 2*a*). Hot monomers interact only with cool monomers and are not in contact with each other in the crystal lattice. The crystal is thus organized as a parallel array of native dimer helices that are interconnected through hot monomer lattice interactions. This situation raises the possibility of systematic disordering of the hot monomers relative to the cool monomer arrays aligned along the *c*-axis crystallographic screws.

Figure 3 plots differences in mean *B*-value ( $\Delta B$ ) between corresponding residues of hot and cool monomers in the native dimer against their difference in radius ( $\Delta R$ ) from the *c*-axis crystallographic screw. This treatment compensates for variations in *B*-value that may arise from the local structural properties of the monomers (for example, extent of local secondary structure) and emphasizes differences that vary systematically relative to the crystallographic screw axis. The observed correlation (0.57) between  $\Delta B$  and  $\Delta R$  for dimer subunits indicates a continuous increase in average temperature factor for residues progressively more distant from the crystallographic screw. This behaviour is consistent with either static or dynamic lattice disordering phenomena involving rotational or wobbling displacements of the dimers relative to the crystal screw axis. The average behaviour (Fig. 3) suggests a maximum *B*-value contribution of 12 Å<sup>2</sup> at the hot monomer periphery resulting from extended lattice disordering effects. Assuming the simplest model involving rigid-body displacement of the dimer about the

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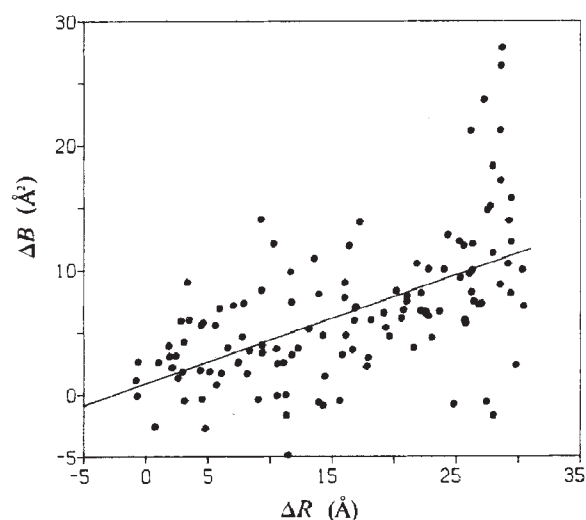
**Fig. 2** *a*, Stereoview of the cytochrome *c'* crystal packing. Subunits (illustrated by  $\alpha$ -carbon backbones) with lower average *B*-values (cool, solid) pack continuously along alternate 2-fold screws (long dashed) parallel to the unit cell (outlined) *c*-axis. Hot (dashed) monomers hold the lattice together by making native dimer (bold) interactions with each cool monomer array and lattice contacts with cool monomer arrays on adjacent screws. *b* Shows how the systematic *B*-value differences between monomers could reflect static or dynamic disordering involving rigid-body displacements of the molecular arrays about the *c*-axis screws. The observed *B*-value difference of  $12 \text{ \AA}^2$  at the hot monomer periphery (Fig. 3) suggests an axial rotation of  $1.2^\circ$  that produces a maximum displacement  $U = 0.7 \text{ \AA}$ , where  $U^2 = 3B/8\pi^2$ . The figure illustrates the superimposed effects of a rigid body rotation about the *c*-axis crystallographic screw that has been exaggerated by a factor of 3 for clarity.



crystallographic screw yields a corresponding r.m.s. amplitude of  $0.7 \text{ \AA}$  (Fig. 2*b*).

The preceding experiment shows that observed differences in subunit *B*-factor behaviour in cytochrome *c'* result from a superposition of localized lattice packing effects that correlate with accessible surface area, and long-range disordering phenomena that reflect the extended pattern of molecular interactions in the crystal lattice. In the present case, the apparent motion may be related to a low-frequency vibrational mode of the V-shaped dimer, as suggested by the correlation (0.40) of monomer *B*-values with distance to the molecular dyad axis. Such concerted molecular flexing could produce the observed overall *B*-value behaviour, assuming that the cool monomers are relatively constrained in their aligned orientations along the *c*-axis crystallographic screws.

The comparison of protein structures determined in different crystal environments provides a basis for correcting *B*-values to account for the localized immobilizations that occur at crystal contacts<sup>4,5</sup>, and thus allows estimates of protein dynamic flexibility in solution. Nevertheless, it is clear that extended lattice disordering effects, which may be unrelated to protein solution dynamics, can make comparable contributions to refined crystallographic *B*-values. It is anticipated that similar effects could occur in other high-resolution crystal structure determinations. However, such lattice effects could go unrecognized in the general case where the crystal asymmetrical unit contains only a monomer. In this case, the potentially non-uniform contributions to *B*-values from lattice disordering would be erroneously



**Fig. 3** Plot of the difference in mean backbone *B*-value ( $\Delta B$ ) between corresponding residues of the cytochrome *c'* subunits against their difference in radial distance ( $\Delta R$ ) from the crystal screw on which the cool monomers are aligned. The least-squares line suggests that the component varying systematically with  $\Delta R$  contributes up to  $12 \text{ \AA}^2$  to the *B*-values of residues furthest from the crystal screw. Large deviations from the least-squares line correspond to residues forming different crystal contacts in the two monomer environments.



attributed to localized protein flexibility in solution. The significance attached to protein *B*-values, both in theoretical studies of protein dynamics<sup>1</sup> and practical applications of medical importance<sup>2,3</sup>, justifies continued efforts at developing new theoretical and crystallographic refinement methods that can simulate crystal lattice effects.

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## Yeast heat-shock protein of $M_r$ 48,000 is an isoprotein of enolase

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Exposure of cells or tissues of various living organisms to elevated temperatures induces the synthesis of a family of specific proteins called heat-shock proteins (HSPs)<sup>1-3</sup>. This phenomenon has so far been investigated mostly with respect to the induction mechanism of these HSPs<sup>4,5</sup>. However, little is known about the function of such proteins, although it has been suggested that they are involved in the acquisition of thermal tolerance<sup>3</sup>. We have recently suggested that a specific class of HSPs may function as negatively regulatory molecules in the growth of eukaryotic cells<sup>6-8</sup>, and that a HSP of relative molecular mass ( $M_r$ ) 48,000 (HSP48) from the yeast *Saccharomyces cerevisiae* may be involved in both thermal tolerance and growth control in this organism<sup>8</sup>. We now present evidence that *S. cerevisiae* HSP48 is an isoprotein of a glycolytic enzyme, enolase (EC 4.2.1.11). This unexpected finding may provide new insight into the role of the protein in the acquisition of thermal tolerance and growth control.

In *S. cerevisiae*, 6 out of 13 HSPs, including HSP48, are durably induced when cells are induced to enter the resting state of the cell cycle,  $G_0$  (ref. 7). These  $G_0$  yeast cells are much more resistant to heat shock than are growing cells, probably because of the accumulation of some or all of the above six HSPs. Induction of certain HSPs was also found with cultured cells of higher eukaryotes when they were arrested in  $G_0$  (ref. 7). Furthermore, we have isolated a heat-shock-resistant mutant of the yeast which is defective in the *HSR1* gene and which constitutively synthesizes six proteins, including two isoforms of HSP48 (ref. 8). The *hsr1* mutant reveals unusual properties involved in growth control, in addition to the selected property that it is ~1,000-fold more resistant to lethal heat shock than the parental *HSR1* strain<sup>8</sup>. Because HSP48 is the only protein that is commonly induced in both growing *hsr1* cells and pre-heated or  $G_0$ -arrested *HSR1* cells, all of which are highly heat-shock resistant, we believe that HSP48 is responsible for the heat-resistance associated with these cells<sup>8</sup>. This is compatible with previous findings indicating that neither the products of the amplified *HSP90* genes<sup>9</sup> nor those of the two cloned genes of the *HSP70* family<sup>10</sup> are directly involved in the acquisition of resistance to lethal heat shock.

**Table 1** Physicochemical and biochemical properties of HSP48 and yeast enolase

|                                                  | HSP48  | Enolase |
|--------------------------------------------------|--------|---------|
| Sedimentation coefficient ( $s_{20,w}^{\circ}$ ) | 5.3S   | 6.0S*   |
| Stokes radius (Å)                                | 36.5   | 36.2†   |
| $M_r$ from $s_{20,w}^{\circ}$ and Stokes radius  | 90,000 | 96,000  |
| $M_r$ from sedimentation equilibrium             | 84,000 | 88,000* |
| $M_r$ from SDS-PAGE                              | 48,000 | 48,000* |
| Enolase specific activity                        | 69 ± 1 | 68 ± 1  |

Enolase activity was determined by monitoring the increasing rate of absorption at 240 nm at 25 °C as a result of conversion of D(+)-2-phosphoglyceric acid (PGA) to phosphoenolpyruvate (PEP)<sup>20</sup>. Standard assay buffer consisted of 50 mM Tris-HCl (pH 7.8 at 25 °C), 2.7 mM magnesium acetate, 0.01 mM EDTA and 1.2 mM PGA. Specific activity is defined as the conversion of 1 µmol PGA to PEP per min per mg protein. Yeast enolase was a product of Oriental Yeast Co. Ltd, Tokyo.

\* Data from ref. 21; † data from ref. 22.

**Table 2** Amino-acid composition of HSP48 and yeast enolase

|     | HSP48 | Yeast enolase |
|-----|-------|---------------|
| Asx | 11.7  | 11.6          |
| Thr | 4.8   | 4.7           |
| Ser | 6.5   | 7.2           |
| Glx | 8.2   | 7.9           |
| Pro | 3.5   | 3.5           |
| Gly | 8.8   | 8.6           |
| Ala | 13.3  | 12.8          |
| Val | 7.4   | 7.9           |
| Met | 1.1   | 1.2           |
| Ile | 5.3   | 5.1           |
| Leu | 9.7   | 9.3           |
| Tyr | 2.1   | 2.1           |
| Phe | 3.7   | 3.7           |
| His | 2.4   | 2.6           |
| Lys | 8.2   | 8.6           |
| Arg | 3.2   | 3.3           |

Values are represented in mol%. Purified HSP48 was resolved in 7.5% SDS-PAGE<sup>23</sup>. The gel was not fixed or stained. Gel slices containing HSP48 were incubated in 0.02% SDS solution (1.0 ml per cm<sup>2</sup> gel) to elute the protein, and the elution was repeated. The combined eluates were extensively dialysed against distilled water for 2 days and lyophilized. The protein was hydrolysed in 6 M HCl at 110 °C *in vacuo* for 22 h and 70 h and analysed in an automatic amino-acid analyser (Hitachi model 835, Hitachi). Amino-acid composition of yeast enolase (peptide 1) was calculated from the amino-acid sequence of this protein deduced from the nucleotide sequence of the complementary DNA<sup>12</sup>. Cys and Trp residues were omitted from the above calculation as we did not determine the content of Cys or Trp in HSP48.

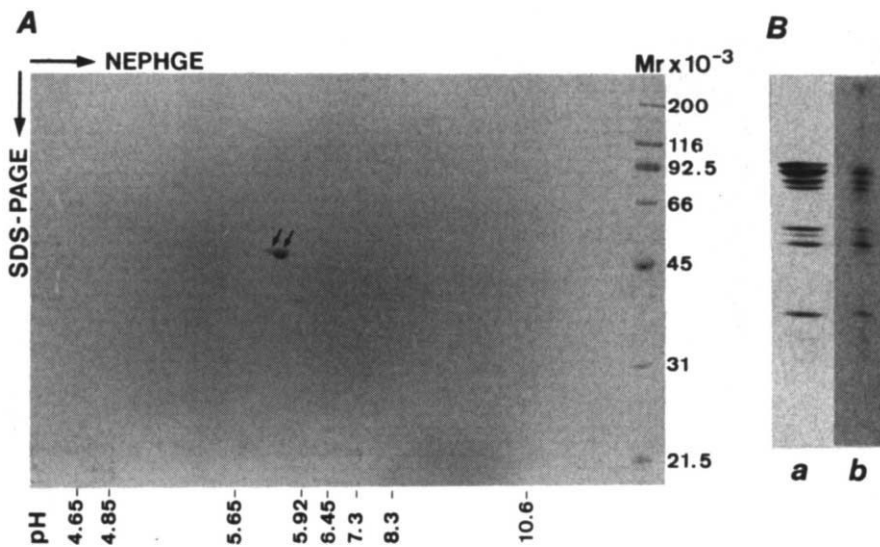
To elucidate the function of HSP48, extracts of yeast cells in the stationary phase underwent four successive purification steps and HSP48 was purified to homogeneity (see Fig. 1 legend). Purified HSP48 is composed of two isoforms, HSP48A (acidic form) and HSP48B (basic form) (Fig. 1A), as we have shown previously<sup>7,8</sup> by two-dimensional non-equilibrium pH gradient electrophoresis/SDS-polyacrylamide gel electrophoresis (NEPHGE/SDS-PAGE) of <sup>35</sup>S-methionine-labelled cells. Peptide mapping confirmed that the purified protein is identical to <sup>35</sup>S-methionine-labelled HSP48 (Fig. 1B).

We determined the N-terminal 23 amino acids of the purified HSP48 on a gas phase sequencer (Fig. 2). Using the Dayhoff protein sequence bank<sup>11</sup> to compare the sequence with those of other proteins, we found that the N-terminal 23 amino-acid sequence of HSP48 is identical to the corresponding sequence of yeast enolase (Fig. 2). In addition, physicochemical properties (Table 1) and amino-acid composition (Table 2) of the purified HSP48 were essentially the same as those of yeast enolase. Furthermore, the specific activity of the enolase in the purified HSP48 protein was the same as that of the authentic yeast

**Fig. 1** **A**, Two-dimensional gel electrophoresis of purified yeast HSP48 (5  $\mu$ g). **B**, peptide mapping of purified HSP48 (lane **a**) and  $^{35}$ S-methionine-labelled HSP48B recovered from the corresponding spot in a two-dimensional gel (lane **b**).

**Methods.** **A**, Isolation of HSP48: Cells of strain X2180-1A (ref. 6) were grown to stationary phase at 23 °C with aeration in SD10 medium<sup>6</sup> in two 2.5-l batches ( $1 \times 10^8$  cells per ml), after which the cultures were incubated for 24 h in the same conditions. After chilling the cell suspensions, we collected the cells by centrifugation at 4 °C at 5,000 r.p.m., and washed them once with 0.05 M Tris-HCl (pH 7.9 at 4 °C). The pelleted cells were 35 g in wet weight, and were stored at -80 °C until use. Half of the frozen cells were thawed and suspended in 20 ml of buffer A (0.05 M Tris-HCl, pH 7.9 at 4 °C, 0.01 M MgCl<sub>2</sub>, 0.01 M 2-mercaptoethanol, 0.1 mM EDTA, 0.25 M mannitol, 1 mM phenylmethylsulphonyl fluoride), after which they were homogenized at 4 °C with 120 g of 0.5-mm glass beads in a Universal Homogenizer (Nihon Seiki Seisakusho Co., Tokyo) for 3  $\times$  2 min. The homogenate was centrifuged at 5,000 r.p.m. at 4 °C for 5 min, after which the resulting pellet was again homogenized and centrifuged. The supernatants were combined (58 ml) and centrifuged at 35,000 r.p.m. in a Beckman Type 35 rotor (Beckman Japan) for 2 h at 4 °C. The supernatant containing most of the HSP48 was dialysed twice against 2 l of buffer B (0.05 M Tris-HCl, pH 8.8 at 4 °C, 0.01 M 2-mercaptoethanol, 1 mM MgCl<sub>2</sub>, 0.1 mM EDTA, 0.02 M NaCl) for 24 h at 4 °C, and applied to a DEAE-cellulose (Whatman DE52) column (3  $\times$  24 cm) equilibrated in buffer B. HSP48 was recovered in the flow-through fractions in buffer B. The fractions were combined and dialysed twice against buffer C (0.01 M sodium phosphate, pH 5.5 at 4 °C, 0.01 M 2-mercaptoethanol, 1 mM MgCl<sub>2</sub>, 0.1 mM EDTA) for 20 h, and applied to a CM-Sephacrose CL-6B column (1.4  $\times$  20 cm) equilibrated with buffer C. HSP48 was recovered in flow-through fractions. One-tenth of the combined fractions containing HSP48 was loaded onto a Sephacryl S-200 column (1.5  $\times$  75 cm) equilibrated with buffer D (0.01 M sodium phosphate, pH 6.0 at 4 °C, 0.01 M 2-mercaptoethanol, 1 mM MgCl<sub>2</sub>, 0.1 mM EDTA) and eluted at a flow rate of 10 ml h<sup>-1</sup>. HSP48 was eluted in a single peak and found to be purified to 99% homogeneity as judged by SDS-PAGE. The HSP48 content of fractions obtained during the purification was determined by densitometry after one-dimensional and two-dimensional SDS-PAGE. About 15–19 mg of purified HSP48 was reproducibly obtained from 17.5 g (wet weight) of yeast cells in the stationary phase with the recovery of 65%.

Two-dimensional gel electrophoresis: The purified HSP48 was lyophilized, dissolved in the O'Farrell lysis buffer, and resolved by NEPHGE<sup>24</sup>, followed by second-dimension electrophoresis in 11% SDS-PAGE<sup>25</sup>. The gels were stained with Coomassie brilliant blue. *M<sub>r</sub>* (Bio-Rad) and isoelectric point (BDH) markers were used<sup>8</sup>. **B**, Cells grown in the stationary phase in synthetic medium (6.7 g l<sup>-1</sup> Bacto yeast nitrogen base without amino acids, 10 g l<sup>-1</sup> succinic acid, 6 g l<sup>-1</sup> NaOH, 2 g l<sup>-1</sup> glucose) were pulse-labelled with 10  $\mu$ Ci ml<sup>-1</sup> L- $^{35}$ S-methionine (1,200 Ci/mmol, Amersham Japan) at 23 °C for 10 min, and chased for 3 min by the addition of non-radioactive methionine to 0.5 mg ml<sup>-1</sup>. The whole-cell extracts were resolved by two-dimensional NEPHGE/SDS-PAGE.  $^{35}$ S-Methionine-labelled HSP48B, cut out from the corresponding spot in the gel, and purified HSP48 (5  $\mu$ g) were separately subjected to peptide mapping with *Staphylococcus aureus* V8 protease (5 ng for each) according to the method of Cleveland *et al.*<sup>26</sup>. Lane **a**, digested products of purified HSP48 stained with Coomassie brilliant blue; lane **b**, digested products of  $^{35}$ S-methionine-labelled HSP48B revealed by fluorography. We have previously shown that HSP48A and HSP48B give similar peptide mapping results<sup>7,8</sup>.



enolase (Table 1). Lastly, a rabbit antiserum directed against yeast HSP48 reacted to authentic yeast enolase (Fig. 3A). From these results, we conclude that yeast HSP48 is enolase.

Searching for further homology between the partial amino-acid sequence of HSP48 and other proteins, we found that the 23 amino-acid N-terminal sequence of HSP48 and the N-terminal 41 amino-acid sequence of yeast enolase<sup>12</sup> were similar in part to the most extensive homologous region<sup>13</sup> found in *Drosophila* HSP70, yeast HSP70 and in the *Escherichia coli* heat-shock *dnaK* protein (Fig. 2). This partial homology suggests an evolutionary relationship between enolase and the HSP70 family. The possibility that enolase and the HSP70 family share a common function (for example, an interaction with the substrate of enolase) remains to be investigated.

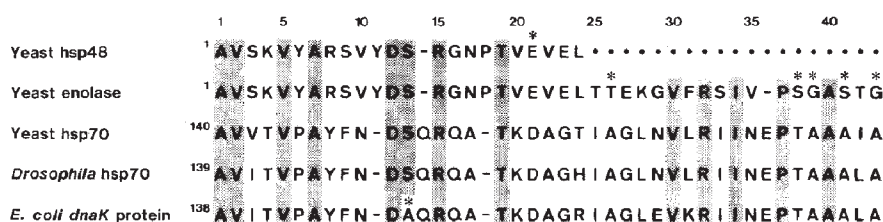
An immune blot analysis of total yeast-cell proteins showed that two proteins, HSP48 and another protein of *M<sub>r</sub>* 47,000 (p47), reacted with anti-HSP48 serum (Fig. 3A). Peptide mapping indicated that HSP48 and p47 are quite similar (Fig. 3B), suggesting that the latter is another isoprotein of enolase. Unlike HSP48, the synthesis rate of p47 was not significantly altered

by heat shock or growth arrest (Fig. 3C). *S. cerevisiae* contains two non-tandemly repeated enolase structural genes, designated *ENO1* and *ENO2* (ref. 12). Enolase 2 polypeptide, the product of *ENO1*, migrates faster than enolase 1 polypeptide, the product of *ENO2*, in SDS-PAGE and the contents of the two enolase polypeptides differ with the growth conditions<sup>14</sup>. We found that the levels of HSP48 and p47 present in cells grown in media containing various carbon sources were quantitatively different (data not shown). Because of this and because of similarities in relative molecular mass, we conclude that HSP48 and p47 are identical to enolase 1 and enolase 2, respectively.

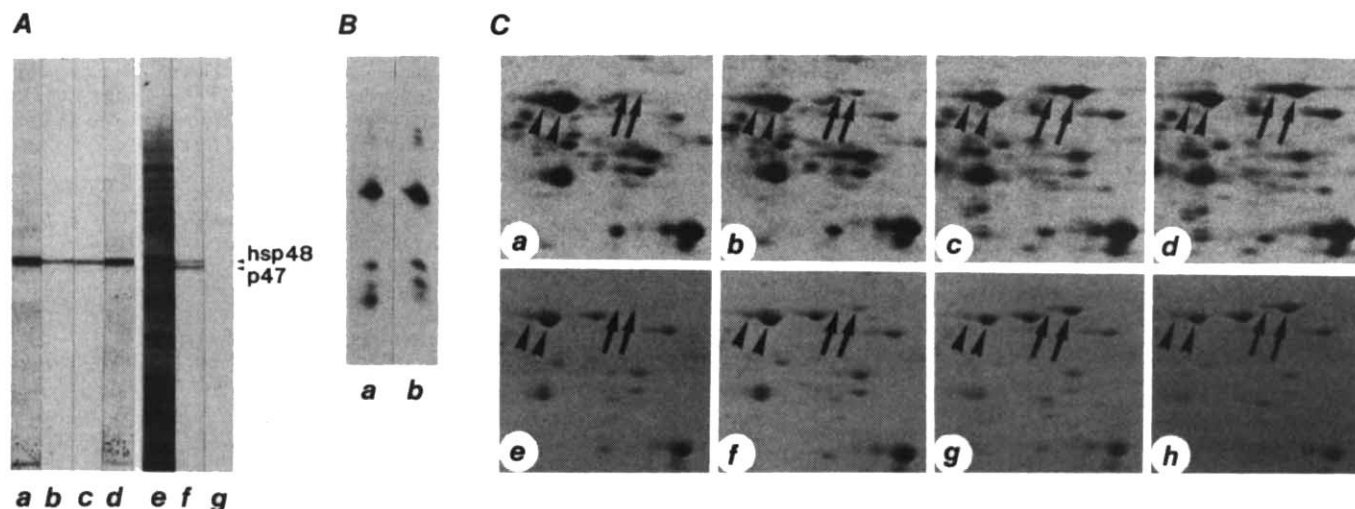
As is the case for HSP48 (HSP48A and HSP48B), p47 is also composed of two isoforms (Fig. 3C). The relative amounts of the two isoforms in each of the two proteins also differ for cells grown in different conditions<sup>7,8</sup>, and it is possible that the enolase activities associated with these isoforms and their intracellular distribution also differ. Thus, the differential expression of the two enolase genes and the differential processing of their products probably have biological significance. Indeed, differential expression of mammalian enolase isoproteins has been observed

**Fig. 2** Comparison of amino-acid sequences of HSP48, yeast enolase and possibly related heat-shock proteins. The sequences of yeast HSP48 (residues 1–23), yeast enolase (Residues 1–41)<sup>12</sup>, yeast HSP70 (the *YG100* gene product, residues 140–180)<sup>27</sup>, *Drosophila* HSP70 (residues 139–179)<sup>28</sup> and *E. coli* *dnaK* protein (residues 138–178)<sup>13</sup> are aligned to show the maximum homology among these proteins. Numbers on the left indicate the position of the residue in the molecule. Shading indicates homology. Asterisks indicate conservative amino-acid substitutions as defined by Schwartz and Dayhoff<sup>29</sup>. Dots indicate residues which have not been experimentally determined. – Indicates a space added to maximize the homology.

**Methods.** An aliquot of purified HSP48 was dialysed against 1 mM ammonium bicarbonate, lyophilized and dissolved in 1% trifluoroacetic acid. Amino-acid sequence results were obtained by using a protein sequencer (Model 470A; Applied Biosystems).







**Fig. 3** *A*, Immune blotting of yeast whole-cell extracts and authentic yeast enolase with anti-yeast HSP48 antibody. Purified HSP48 (lanes *a*, *b*), yeast enolase (lanes *c*, *d*) and extracts of X2180-1A cells in the stationary phase (lanes *e*–*g*) were resolved in SDS-PAGE and electrophoretically transferred to a nitrocellulose sheet. The sheet was stripped and strips were separately incubated with 1:500 diluted rabbit yeast anti-HSP48 antiserum (lanes *b*, *c*, *f*) and preimmune rabbit serum (lane *g*), followed by incubation with peroxidase-conjugated goat anti-rabbit IgG (Cappel). A strip was stained with Auro Dye (Janssen) (lanes *a*, *d*, *e*). *B*, Peptide mapping of HSP48 and p47. The spots of  $^{35}\text{S}$ -methionine-labelled HSP48 (lane *a*) and p47 (lane *b*) were subjected to peptide mapping with *S. aureus* V8 protease as described in Fig. 1 legend. *C*, synthesis rate and content of HSP48 and p47 in heat-shocked or  $G_0$ -arrested yeast cells. *a*, *e*, Exponentially growing X2180-1A cells at 23 °C in synthetic complete medium; *b*, *f*, heat-shocked cells at 36 °C for 1 h; *c*, *g*, cells kept in stationary phase for 24 h; *d*, *h*, cells starved of sulphur for 24 h by incubation in sulphur-free medium<sup>8</sup>. The cells in each of the above cultures were pulse-labelled with  $^{35}\text{S}$ -methionine and chased as described in Fig. 1 legend. The whole proteins (25 µg each) extracted from these labelled cells were analysed in two-dimensional gel electrophoresis as described for Fig. 1. Protein spots were revealed by autoradiography (*a*–*d*) and staining (*e*–*h*). A pair of arrowheads indicate p47A (left; acidic form) and p47B (right; basic form). A pair of arrows indicate HSP48A (left) and HSP48B (right).

during differentiation and maturation of neurones and teratocarcinoma and neuroblastoma cell lines<sup>15–18</sup>.

The expression of the enolase genes seems to be regulated by many systems. First, it depends on carbon sources in the media<sup>14</sup>. Second, we have shown previously that the *HSR1* gene negatively regulates the synthesis of six proteins—HSP48A, HSP48B, two  $G_0$ -induced proteins (p73 and p56) and two unidentified proteins (p63 and p60)<sup>8</sup>. Third, exposure of *HSR1* cells to mild heat shock increases the synthesis rates of the two HSP48s and of other HSPs<sup>7</sup>. Finally, *hsr1* cells starved of sulphur or arrested in the stationary phase synthesize HSP48A, HSP48B, p73 and p56 at higher rates than do exponentially growing *hsr1* cells<sup>8</sup>. These results suggest that the synthesis rate of HSP48 (enolase 1) is affected by at least three factors, the *HSR1* gene, growth conditions and heat shock, although these factors may partly overlap. In agreement with our results, McAlister and Holland have noted that the content of enolase 1, but not of enolase 2, is significantly increased in yeast cells grown to the stationary phase<sup>14</sup>.

Finally, the question arises of whether HSP48 functions solely as a glycolytic enzyme when it is involved in growth control and in protecting yeast cells from lethal heat shock. The synthesis of other glycolytic enzymes linked to enolase, such as glyceraldehyde 3-phosphate dehydrogenase or pyruvate kinase, is not significantly altered in *hsr1* cells or when *HSR1* cells are pre-heated by 36 °C (ref. 19 and our unpublished observation). This result is consistent with the hypothesis that the heat-shock resistance acquired by the *hsr1* mutation and heat pretreatment may be ascribed to some function associated with HSP48 other than enolase activity. Analysis of the intracellular distribution and molecular interactions of HSP48 (enolase 1) with other molecules, especially in comparison with p47 (enolase 2), should provide more direct evidence.

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## Erratum

### Colour, albedo and nucleus size of Halley's comet

D. P. Cruikshank, W. K. Hartmann & D. J. Tholen  
*Nature* **315**, 122–124 (1985).

ON page 122, column 1, line 3 of the first paragraph should contain the values  $J$  (1.25 µm) and  $V$  (0.55 µm).

## Retroviral protease-like sequence in the yeast transposon Ty1

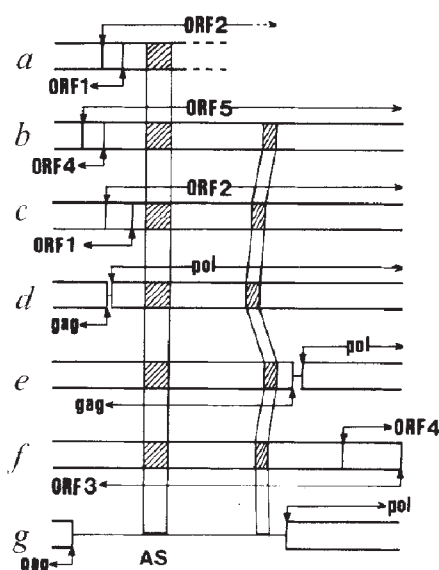
RECENTLY, Mellor *et al.*<sup>1</sup> determined the nucleotide sequence of the 5'-terminal portion of the yeast transposon Ty1 and identified two open reading frames, ORF1 and ORF2, which are in the same relative positions as the *gag* and *pol* coding sequences in retroviruses. In addition, they showed that Ty1 produces a fusion protein by a specific frameshifting process similar to that used by retroviruses. We have shown that retroviruses, cauliflower mosaic virus (CaMV), *Drosophila* copia-like element 17.6 and Syrian hamster intracisternal A-type particle (IAP) contain sequences homologous to the *gag*-specific protease p15 of Rous sarcoma virus (RSV) in the same relative positions<sup>2,3</sup>. Furthermore, these p15-related sequences contain a highly conserved stretch of amino acids which shows a close similarity with sequences around the active-site amino acids Asp-Thr-Gly of the acid protease family, suggesting that they possess a similar activity to that of acid proteases<sup>2</sup>.

A computer-assisted search for homology has revealed the presence of the

**Table 1** Amino-acid sequence comparison between p15-related proteases and pepsin-related acid proteases

|                            |                    |
|----------------------------|--------------------|
| Viral protease             |                    |
| Ty1 ORF2                   | GHLL-DSGASRLIRS    |
| CaMV ORF5                  | LHCFV-DTGASLCIA-S  |
| 17.6 ORF2                  | LKCL-ITGSTVNMT-S   |
| M-MuLV <i>pol</i>          | VTFLV-DTGAQHSVL-T  |
| RSV <i>gag</i> p15         | ITALL-DSGADITII-S  |
| IAP ORF3                   | FEQIM-DSGADKSI-S   |
| HTLV ( <i>gag-pol</i> )    | IEALL-DTGAQMTVL-P  |
| Acid protease (C-terminal) |                    |
| Pepsinogen (human)         |                    |
| (porcine)                  | COAIV-DTGTSLLTG-P  |
| Prochymosin (bovine)       | COAIV-DTGTSLLTG-P  |
| Penicillopepsin            | CQAIL-DTGTSLKLVG-P |
| Renin (mouse)              | FSGIA-DTGTLLLL-B   |
| (human)                    | CEVVV-DTGSFISA-P   |
| Acid protease (N-terminal) |                    |
| Pepsinogen (human)         | CLALV-DTGASYISG-S  |
| (bovine)                   |                    |
| (porcine)                  | DFTVVFDTGSSNLWV-P  |
| Prochymosin (bovine)       | DFTVIFDTGSSNLWV-P  |
| Penicillopepsin            | DFTVIFDTGSSNLWV-P  |
| Renin (mouse)              | EFTVLFDTGSSDFWV-P  |
| (human)                    | TLNLFDTGSDLWV-F    |
|                            | TFKVMFDTGSANLWV-P  |
|                            | TFKVMFDTGSSNVWV-P  |

As pepsin-related proteases consist of two topologically similar domains and two active-site aspartic acids, the amino-acid sequences around the two active sites were aligned between the N- and C-terminal halves. Bold type indicates amino acids that are identical or chemically similar among 18 sequences (90%) out of 20 at each position. Gaps (-) were inserted to increase sequence similarity. \* Active sites of acid protease family. For sequence data, see refs 2 and 3.



**Fig. 1** Maps of homology between protease regions of reverse transcriptase-containing viruses and transposons: *a*, Ty1; *b*, CaMV; *c*, 17.6; *d*, M-MuLV; *e*, RSV; *f*, IAP; *g*, HTLV-I (human adult T-cell leukaemia virus). Homologous segments are linked by slanted lines. No open reading frames are assigned between *gag* and *pol* in HTLV-I. Data were taken from ref. 2 for *b-e* and *g*, and from ref. 3 for *f*. AS, the region showing a close sequence similarity with active-site amino acids of the acid protease family.

p15-related sequence in the ORF2 of Ty1 (Fig. 1, Table 1). Thus, the N-terminal portion of the ORF2 product may have an activity similar to that of acid proteases. Such a widespread occurrence of the protease sequence suggests that it is vital for these viruses and transposons. Mellor *et al.* suggested that the fusion protein is processed proteolytically<sup>1</sup>; the ORF2-coded protease may be involved in this processing. The relative positions of the conserved segments are almost the same among Ty1, CaMV, 17.6 and Moloney murine leukaemia virus (M-MuLV) (Fig. 1), suggesting that the observed homologies are the result of divergence, not convergence. This also supports the notion that the ORF2 corresponds to *pol* in retroviruses<sup>1</sup>, and thus the reverse transcriptase coding sequence, which is highly homologous among reverse transcriptase-containing viruses and transposons<sup>2-5</sup>, probably lies in a region immediately downstream from the protease region.

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**MELLOR ET AL. REPLY**—We thank Toh *et al.* for their interest in our paper<sup>1</sup> and for pointing out the homology with acid proteases at the beginning of ORF2 in Ty1-15. We would like to add that in pulse-chase experiments the protein, p1(Ty1-15), encoded by ORF1 in Ty1-15 seems to be processed, presumably by proteolytic cleavage. Furthermore, we have recently identified a candidate for the 'natural' product of ORF1/ORF2 fusion. This protein, p3(Ty1-15), has a relative molecular mass of 190,000 and can be seen only in short pulse-labelling experiments. Like p1(Ty1-15), therefore, it also appears to be proteolytically cleaved. We have observed four possible cleavage products and their relationships with p1(Ty1-15) and p3(Ty1-15) are presently being established. The 'processing' of p1(Ty1-15) is dependent on the expression of ORF2, a result that is consistent with the processing protease being encoded within this region and therefore in agreement with the



observations of Toh *et al.* The interrelationships of Ty proteins will be reported elsewhere<sup>2</sup>.

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## Forest succession

MOST forest ecologists will agree with the conclusion of Finegan<sup>1</sup> that in real forests, succession commonly has characteristics of both the contrasting models he has described: a 'holistic' facilitation model where successive species replace each other and a 'reductionist' model where all species arrive at the outset.

Finegan is wrong, however, to say that no direct studies have been made of forest succession. Many studies do indeed rely on contemporaneous observations of forest patches of purportedly known age and history, but in the Eastern tropical rainforests there have been four studies of forest patches through time which clearly illustrate various departures from the two paradigms. These studies, originally reported in the specialist forestry literature, were used in the analysis of dynamic processes in a general account of the Eastern rainforests<sup>2</sup>.

At Kepong, Malaysia, forest on an abandoned field has been monitored since 1947<sup>3</sup>. Here, the flora changed little between 1949 and 1960 because of a fern blanket, which was eventually shaded out, so from 1960 to 1976 there was a marked change, though climax species were still scarce. At Sungai Kroh, Malaysia, a similar succession was monitored for 17 years from 1946 to 1963<sup>4</sup>. Here, some tree species, including the dominant species, were present throughout while others progressively arrived. No fern blanket developed. At Sungai Menyala, Malaysia, a secondary forest established in 1917 has been monitored since 1947<sup>2,5</sup>; here spasmotic floristic change is still occurring. After natural catastrophic destruction by repeated cyclones, succession at Kolombangara in the Solomon Islands has been under observation since 1964, and analysed for the first 6.6 years<sup>2,6</sup>. The latter two studies also include areas of 'climax' forest which are showing 'cyclic replacement'<sup>7</sup> (rather than 'directional change in time' as Finegan<sup>1</sup> defines succession) much more rapid than suggested by con-

ventional wisdom about the stability of primary equatorial forests. Finegan excluded cyclic replacement from his discussion. It does not involve directional change in time and is the process whereby climax ecosystems are maintained in the absence of catastrophic destruction. (The latter is followed by 'secondary' succession (as it is usually termed) back to climax.) Cyclic replacement is occurring at Sungai Menyala.

At Kolombangara island, the north-coast forests are believed to be repeatedly destroyed by cyclones; they show succession as discussed by Finegan. The west-coast forests, by contrast, are outside the influence of cyclones and show cyclic replacement. Sungai Menyala and Kolombangara demonstrate the value of long-term studies, and in fact these studies have continued longer than any others in climax tropical forest. The Kolombangara study includes the monitoring of recruitment, growth and mortality of seedlings of the 12 main large tree species and how these are influenced by different amounts of leafy canopy overlying them. For these species the total population is being monitored and the resultant understanding of their autecology has aided the analysis of the contrasting dynamics of successional (north-coast) forests and cyclically replacing (west-coast) forests. So far, nine sets of observations over 6.6 years have been analysed, and a further analysis is planned to start in 1986, covering another three sets of observations to 21 years. Both the Kolombangara and Sungai Menyala forests are under threat of conversion to more lucrative land-use, the much-discussed fate of many tropical rainforests, despite the contribution they have made to ecological understanding of forests<sup>2,8–11</sup>, which itself underpins silviculture. The longer the studies continue, the more they reveal.

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FINEGAN REPLIES—I thank Whitmore for pointing out this gap in my review<sup>1</sup>. The studies he cites are interesting and illuminating and deserve a wider audience

(although I was unable to find Whitmore's ref. 5 and am unable to comment on it).

One point concerning long-term studies is worth making here. Two of those cited by Whitmore<sup>2,3</sup> provide structural and floristic description only, and therefore cast very little light on the mechanisms of the successions which are clearly occurring. Measures of species abundance against time can be used to support almost any mechanistic model of succession, from the initial floristic composition ideal to a number of composite processes, as shown in my Fig. 4<sup>1</sup>. No amount of description, whether inferred or obtained directly, will overcome this problem; hence the last paragraph of the review. Whitmore's studies at Kolombangara exemplify the sort of approach which is needed<sup>4</sup>.

I did not discuss cyclic replacement because there is a clear ecological basis for separating this process from the successions I review, involving differences in initial environmental conditions and sources of colonizing individuals. As Whitmore himself has pointed out, pioneer trees participate only infrequently in cyclic replacement patterns<sup>4,5</sup>. I should have restricted my formal definition of succession by referring to processes occurring in cleared areas large enough to permit the establishment of a vegetation dominated physiognomically by pioneer trees.

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## Matters Arising

Matters Arising is meant as a vehicle for comment and discussion about papers that appear in *Nature*. The originator of a Matters Arising contribution should initially send his manuscript to the author of the original paper and both parties should, wherever possible, agree on what is to be submitted. Neither contribution nor reply (if one is necessary) should be longer than 500 words and the briefest of replies, to the effect that a point is taken, should be considered.

# Thinking about thinking

Duncan Davies

## Science as a Commodity: Threats to the Open Community of Scholars.

Edited by Michael Gibbons and Björn Wittrock.

Longman: 1985. Pp. 173. Pbk £9.95, \$15.

"SHOULD university teachers or researchers display patriotism by undertaking work for the armed forces which will inevitably be secret and unpublishable?" "Should they help pay for their institutions in hard times by making contracts with industrial firms whereby publication of research must be delayed, perhaps for a year or two, until intellectual property rights have been safeguarded by patents?" "Is public funding of university work best guided by simply choosing the best men, and leaving them to choose their research topics as best they can?" "Would the best women, if so funded, choose differently from the best men?" "For such privileges, who are the best people and how do you choose them?"

These are good questions, the resolution of which is of great importance for the maintenance of democracy and survival of freedom of inquiry and speech. It is therefore eminently reasonable to convene a conference to discuss them in detail. One would invite people from a cross-section of backgrounds: university teachers currently doing research in the humanities, engineering and science (and, if possible, across more than one field); practising men of business and public affairs; and some military people, to relate their problems and experiences. One would cover diplomacy, matters of public order, economics, investment, taxation and technology, with science and philosophy bulking large in the background. There would doubtless be difficulties of language and psychology, and many important differences in priorities and chosen compromises, but the result would be very fruitful.

Conversely, it might seem one-sided to conduct such a conference in the virtual absence of the practitioners of science, and to publish essays only by (admittedly able and clever) university teachers whose specialism is mainly the study of those who study engineering and science, in the absence of practitioners of finance, law, psychology and medicine. And it might seem confusing to use the title *Science as a Commodity*.

There used to be a gibe that you could always find the Germans in a crowd by inviting everyone to choose between two doors marked "Heaven" and "Lectures on Heaven". Excessive specialist theorizing is now spread throughout the Anglo-Saxon world, to the extent that it is no longer fair to pick on the Germans. Those of us who have worked hard to encourage

interest in broader matters in the choice, conduct and exploitation of research, feel a little frustrated because success in drawing attention to this synthesis has created another band of specialists, engaged not in practice, but only in a new branch of theory.

There are wise thoughts in this book, the published record of the meeting, but there is also an overdose of theology, and repetitious concern with a few causes celebres (such as Faust, Bacon, the Manhattan Project — described rather inaccurately by one of the contributors — and the arrangements between Hoechst and Mr Whitehead on the one hand, and the universities in Boston, Massachusetts, on the other). And there is very little about the actual products, processes and services which most illuminate the connection between free inquiry and practical application (or which comes first), or whether the great increase in society's investment in technology seems to have helped or hindered such basic research as the astronomical study of the Universe, its cosmological interpretation, the structure of matter and energy, and the origins of life and inheritance. It may be that the practical men (inside and outside the universities) were in fact invited but would not come; if so, their contributions should have been sought by other means, and not beaten into shape with theory.

The unconfused title for the book could well be *Technology as a Tradeable Investment* or *Should Scientists do Secret Military Research?*, but these would not sound sufficiently mysterious and profound. Professor Wittrock, in his summing up on p. 156, says "Science has not become a commodity; it always was". Professor Salomon, on p. 78, says "Science as a commodity? Only half a century ago, such a question would have appeared as a provocation, and a century ago, as an aberration". This apparent disagreement between gurus stems, of course, from different interpretations of the word "science", and the problem is intensified for two other reasons: first, the words science, technology and research are repeatedly used interchangeably. Secondly, in this context the term "commodity" is less appropriate than the term "investment". The trading of investments is a rather different process from trading in consumer goods. Technology, rather than science or research, is our most comprehensible kind of intellectual investment. It has been traded always, but most particu-

larly since the patent system was introduced four centuries ago to *prevent* (not assist) secret operation; the method was the award of a temporary monopoly in return for disclosure, to allow the recoupment of the investment cost. Inventive applied science can sometimes be traded as a component of technology, but the ways of nature cannot be closed to astronomers, high-energy physicists or geneticists. These subjects can be kept secret only for as long as there is military control over the instruments needed for the work (rockets to launch the satellite: detectors to measure the radiation). By contrast a commercial firm would want to go public and sell the gear.

So the real problem is that of the *military* restraint of the dissemination of *technology*, with its attendant economic penalty for all, and not that of the selling of university science by commercial interests. Commerce requires the most temporary protection of knowledge that will make long-term research and development worth while, and the earliest practicable publication. Men of good will such as Sir Cyril Hinshelwood, President of the Royal Society from 1955 to 1960, regarded applied research, of whatever kind, as one of the most valuable sources of ideas for scientific discovery; they sought to participate in both discovery and invention. When military secrecy hindered this natural exchange, they sought means for distilling the clues for science into publishable form.

Once this confusion is cleared up, the book becomes a useful quarry with some interesting thoughts on a variety of matters. For instance it is hard not to agree with Helga Nowotny that women would choose different research topics from men, and hard not to conclude that both science and technology are impoverished by the small number of influential women involved. But the real need is for much more study of individual examples, for example to help decide whether peacetime science and the technology-based business that funds it depends heavily, lightly or not at all on science conducted for military purposes. Did polythene, radar and the gas turbine (all invented in peacetime) require wartime funding to reach peacetime use? Was anything at all invented in wartime; does war merely accelerate and enable the development of peacetime ideas that were languishing? More broadly, would cephalosporin have been discovered by a firm, and could the semi-synthetic penicillins have been developed in a university? More pragmatism, please, before the next bout of analysis. □

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## Awash in an ocean of information

David Smythe

### **The Geology of the Atlantic Ocean.**

By K. O. Emery and Elazar Uchupi.  
*Springer-Verlag: 1984. Pp.1,050. DM 360, \$104.80.*

THE Atlantic Ocean has grown a few centimetres wider in the past few years. It has also undergone a change in location. The North Atlantic is nowadays thought of as the segment between Europe and Greenland, and the Africa – North-America segment is now the Central Atlantic. The South Atlantic remains as before, between Africa and South America. This sensible shift in nomenclature avoids such possible infelicities as "southern Central North Atlantic", or even "northern Northern North Atlantic". Emery and Uchupi's encyclopaedic compilation, some eight years in the making, sticks to the older (oceanographic) rather than the new (plate tectonic) Atlantic, and barely reaches Iceland. The book is therefore incomplete, which is a pity; many of the current problems in understanding the complex plate history north of 50°N or so have arisen through an artificial division of expertise and interest north and south, respectively, of the Greenland–Scotland Ridge.

Eleven pairs of large, loose-leaf charts cover the region 60°N to 60°S. They include bathymetric, physiographic, sediment isopach, and tectonic maps. Each chapter is ocean-wide in scope, ranging from history, through physiography, geology and evolution, to a final chapter on geography and economics, including waste disposal and hydrocarbons. The most up-to-date and comprehensive of them are the accounts of syn-rift and drift sediments — subjects on which the authors are long-established authorities. These chapters, making up some 40 per cent of the book, draw upon many references of 1980–1983 vintage, and take advantage of good-quality reproduction or re-drawing of original figures.

Where the discussion strays beyond the book's title — both sideways and downwards — I have increasing reservations. Some sections have a definite air of obsolescence. Why, for example, write about the North Sea at all if you have to lean heavily on review papers, one or two of which were mediocre even when they were read at the first major conference on

the North Sea in 1974? Why try to write about the Neolithic and Early Bronze Age sailors of north-west Europe (incorrectly referred to as Celts) if you neglect their knowledge of navigation? They were able to predict complex lunar motions and presumably, therefore, the powerful Atlantic tides on which their maritime civilization depended. The North Sea has become a mature oil province since the early 1970s, and in the same period the whole new science of archaeoastronomy has blossomed.

The chapter on internal igneous structure begins at the core and works upwards through geomagnetism, the mantle, heat flow and seismicity, petrology and so on to the ocean crust itself. On the way up there is a short excursion to the Moon. The heart of the subject — the structure, petrology and magnetic properties of oceanic crust — is dealt with in rather cursory manner; for example, there are no diagrams here showing the great variety of evolutionary models that have been thought up for the basaltic Layer 2.

Even more unfortunately, an important section on the geomagnetic polarity reversal timescale picks upon one of the least satisfactory revisions for the late Mesozoic and Cenozoic, which was published in 1980. An entire industry of reversal time-

scale "improvements" has sprung up in the United States in the past few years, but Emery and Uchupi perpetuate an inapplicable omission by the specialists of any reference to the superb work of Hailwood and co-authors, which appeared in the Initial Reports of the Deep Sea Drilling Project in 1979 — hardly an obscure source. Six years ago you could have taken Hailwood's scale, added it to the Gubbio timescale for the late Cretaceous, run it through a pocket calculator to update the radiometric decay constants, and you would have had a "working" timescale for the past 100 million years or so, essentially as good as any of today's efforts. The moral of this example seems to be that you have to publish loud, fast and often in order to get cited, which will merely hasten the day when we are all drowned by the rising sea-level of publications.

Emery and Uchupi have obviously had difficulty in keeping their ambitious project watertight in the decade since they laid its keel. Although they have made a valiant effort, I think that the scope of the subject, at least in the terms that they see it, is now simply too broad for any two people to tackle successfully. □

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## BWP four

John Andrews

### **Handbook of the Birds of Europe, the Middle East and North Africa. The Birds of Western Palearctic. Vol. IV: Terns to Woodpeckers.**

Chief editor Stanley Cramp.  
*Oxford University Press: 1985.*  
*Pp.960. £60.*

THE latest volume of "BWP", as it has come to be known, describes a further 113 species of birds from the 760 or so which occur in the Western Palearctic. Commencing with the terns, skimmers and auks, it progresses through sandgrouse, pigeons and parrots — whose sole representative is the introduced ring-necked parakeet — to cuckoos, owls, nightjars, swifts, kingfishers, bee-eaters, roller and hoopoe, concluding with the woodpeckers.

Each species entry runs to about 6,000 words. As well as giving field identification characteristics, it provides detailed descriptions of plumages necessary for the identification of specimens in the absence of reference skins, and data on moult measurements, weights, structure and geographical variation. The colour plates, all of them by artists with field experience of most if not all of the subject genera, show the main plumages, including downy young (and eggs) with an acceptable consistency of style.

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Once the text moves on to material derived from research it is inevitably more varied in quality and comprehensiveness. The habitat descriptions enable one to look for a given bird in the right place but are often insufficient, even when considered together with information on food, to guide either habitat management or conservation judgements on the implications of subtle changes in land use. In general, more is known of breeding requirements than of wintering habitat usage, and that too is reflected in the book.

Distribution maps show separately the world and Western Palearctic ranges, while the text concentrates upon historic changes in range (which yet often remain inexplicable). Population size is usually based on national estimates, which are notoriously questionable.

The bulk of the text is devoted to social patterns and behaviour, and breeding biology. In bringing together all of this material, the book — and the series — has value not only in its own right but as a basis for comparison which may in time extend our knowledge of "why" as well as "what". By implication, this massive and invaluable exercise in ornithological publication raises the question of whether the work should be allowed to end with the appearance of each volume or whether some continuing process of updating is practicable and worthwhile. □

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### **New journals review**

On 26 September *Nature* will publish the fifth annual review supplement devoted to science journals. Publishers and learned societies wishing to submit material should refer to last week's issue of *Nature* (p.607) or contact the Book Review Editor (London 836 6633).

## Culture of genetics

Peter Goodfellow

### Mammalian Cell Genetics.

By Martin L. Hooper.

Wiley: 1985. Pp.175. £63.25, \$49.95.

AT THE same time as the molecular revolution in genetics, a quieter revolution has occurred in the genetics of cultured cells. Older techniques, such as variant isolation and production of somatic cell hybrids, have been improved and supplemented with methods such as DNA-mediated gene transfer, expression of bacterial genes in mammalian cells, microcell transfer of chromosomes and chromosome-mediated transfer of sub-chromosomal fragments. In *Mammalian Cell Genetics* Dr Hooper has provided a commentary on the whole of modern somatic cell genetics, designed to be read in conjunction with the primary literature.

Guides such as this can either be Talmudic, expanding and clarifying an obscure text or, like the synopsis of an opera, condense the story while emphasizing the highlights. By the author's stated intention, the book falls into the latter category. It does not purport to tell you how to do an experiment but informs you of the experiments that have been done. As such it will provide novices with an overview of the possibilities available before they delve into more specialist publications.

A good commentary should be comprehensive and emphasize the right points. *Mammalian Cell Genetics* is comprehensive for techniques up to 1983, but the tables of cell lines available should not be

regarded as exhaustive and events of the past two years have somewhat overtaken the tables of molecular vectors. The choice of highlights is more open to argument and largely a matter of personal opinion. I would have emphasized human gene mapping, monoclonal antibodies and isolation of oncogenes by DNA transformation of cultured cells. Perhaps as a consequence of his apparent determination to be succinct, Dr Hooper treats the first two of these achievements as derivatives of the esoteric, and often arcane, truths of chromosomal segregation and extinction of "luxury" functions in hybrid cells. Formally he is correct, but, for me, it counts as a failing. Limitations on length probably also explain the absence of some essential practical details, such as the amount of transforming DNA a mammalian cell can take up and incorporate into its genome or the maximum size of individual DNA molecules which can be incorporated. Also missing is debate about the major unsolved problems in mammalian cell genetics; for example, many people would like to obtain homologous recombination between exogenously introduced DNA and the equivalent recipient cell gene.

The book is a helpful guide and any postgraduate student of mammalian genetics would benefit from reading it. Unfortunately, the absurd price means that our hypothetical student will either have to spend a week's wages or read somebody else's copy. I would suggest the second alternative. □

*Peter Goodfellow is Head of the Human Molecular Genetics Laboratory at the Imperial Cancer Research Fund Laboratories, London.*

## Colourful behaviour

M. E. G. Evans

### Insects in Camera.

By Christopher O'Toole.

Oxford University Press: 1985. Pp. 154. £14.95, \$29.95.

THIS is not just another book of pretty pictures of animals. Rather, it is a collection of superb colour photographs by Ken Preston-Mafham in which are captured the behaviour of living insects. Christopher O'Toole's text introduces and explains the photographs so that we have a rather unusual combination of linked illustrations and text, describing not merely a series of insect types but their great diversity of behaviour patterns.

The question posed is "How are insects adapted to survive in a hostile world?". The answer, of course, is "very well", but this book delves into the underlying reasons. There are seven sections, each of which is introduced by a short essay. The rest of the text consists of long legends to

each of the photographs, in which the species and their systematic positions are noted, and their activities described. The photographs are not just of insects seen against typical backgrounds; some are unique, for instance the picture of a hovering muscid fly introducing her offspring into driver ant society by the precision bombing of an ant column with her eggs.

A disadvantage of breaking up the text into a series of short paragraphs is that the book is necessarily somewhat fragmented; there are nearly as many stories as there are pictures. However, my main criticism is that where there are three or four prints to a page the detail cannot be appreciated. I would have preferred none of the photographs to have been less than half-page size, even at the price of fewer of them. Nevertheless, this is a stimulating account of insect behaviour and adaptation, with an intelligent text elaborating upon the excellent pictures. For many readers it will be literally an eye-opener. □

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# Personal atmosphere for the physicist

J. R. Milford

## Physical Meteorology.

By Henry G. Houghton.

MIT Press: 1985. Pp.442. \$37.50 £41.95.

FOR Professor Henry Houghton's colleagues and former students the publication of *Physical Meteorology* will be an event of some note. It will also enable a much wider range of atmospheric physicists to benefit from his wisdom. The qualities of a great teacher shine through this book, with scholarship, clarity of argument, enthusiasm and a welcome readiness to discuss points of uncertainty. He is also ready to say when an exact theory is inapplicable to a real, untidy atmosphere, for instance when we consider the scattering of radiation.

The use of the book as a text to support other people's courses will be restricted, however, by a number of its characteristics. First, it is only for competent physicists, assuming as it does some prior knowledge of Raman scattering, or of what is meant by local thermodynamic equilibrium, to give just two examples. Secondly, the choice of material is very personal, as indeed is made clear in the preface. The main sections consist of four chapters on radiation in the atmosphere and three on cloud physics: these are preceded by an opening chapter on the atmospheric aerosol. This is logical enough, but there is only minimal explanation as to how this information may be used later in the book and there are in fact few references back to it. The final chapters on optical phenomena and atmospheric electricity are also self-contained.

The book thus covers the topics which are traditional in texts on atmospheric physics. Professor Houghton admits to doubts on the title, and it seems to me that *Physical Meteorology* is now a misnomer: operational meteorologists will not find much of the theory behind their practice here. The turbulent transfers of energy which bulk large in much of our science find no mention, and surely the links with large-scale energetics merit discussion if meteorology is included in the title?

In smaller matters the choice of material is again unashamedly (and excusably) personal. However, more applications of the existing material could have been in-

cluded: illustrations of scattering or anomalous propagation of radar would have helped readers to make links with specialist studies without any risk of trespassing on them. The relevance of the radiation chapters to remote sensing could have been brought out too, in contexts other than temperature sensing with the  $15\mu\text{m}$   $\text{CO}_2$  band. Even in this section the paragraph on the non-American method is so brief as to be quite unhelpful.

Textbooks which include problems at the end of each chapter are always attractive to other teachers, but those included here are less useful than some since they are mainly designed to stimulate open-ended discussions. They provide no help to the struggling lecturer who needs to know whether he has got it right.

As I read the book I was occasionally

irritated by misprints, and, more often, by the adherence to old-fashioned units, even when a change would be trivial: why not replace  $^\circ\text{C}^{-1}$  by  $\text{K}^{-1}$ ? Also, the basic radiation and derivations are not entirely clear, mainly due to Professor Houghton's refusal to use the term "radiance". Some diagrams raise questions I would like answered, such as Fig. 7.14 which includes collision efficiencies for drops rising to a value of ten in a very curious fashion. But one should not be put off by such deterrents, or by the absence of references after 1980, for here there is much insight and illumination of the principal parts of atmospheric science. □

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## Psychological notes

Eric Clarke

### The Musical Mind: The Cognitive Psychology of Music.

By John A. Sloboda.

Clarendon: 1985. Pp.291. £20.

THE power of music to move people's minds, and the ability of a few minds to produce performances and compositions of great profundity, have raised questions that can be traced from Plato's *Republic* to the current Oscar-award-winning film *Amadeus*. Since the beginnings of modern psychology they have also been a focus of attention for those attempting to understand musical cognition with more rigorous methods. Until recently the psychology of music could not claim to be a major area of research, but two new journals on the subject and a steady stream of books and articles indicate that this is changing.

Most of the literature has, however, held more interest for the experimental psychologist than for the musician, concerned as it is with highly reduced "musical" materials investigated under extremely constrained (and unmusical) conditions. It is therefore a breakthrough that a book has now appeared which brings together recent work in a way that demonstrates its significance for musicians, whilst in no way compromising the psychological theory on which it is based.

In the preface to *The Musical Mind*, John Sloboda makes it plain that the primary aim of his book is to bridge this gap, and he is to be congratulated on doing so. Sloboda's cognitive approach, and his desire to address matters that preoccupy musicians, have resulted in a book that covers many areas usually absent from writings on the psychology of music. Thus while there is a substantial chapter on listening to music, there are also accounts of musical learning and development, musical performance, and compo-

sition and improvisation. The section on composition is an excellent attempt to unravel the psychological processes involved in this least-understood area of musical cognition, using the compositional sketches of various composers (Beethoven, Mozart, Stravinsky), and the written accounts of Sloboda's (and another composer's) compositional methods. It is Sloboda's willingness to make use of evidence from a wide variety of sources that allows him to delve into areas previously considered too private to be investigated, and which brings an engaging freshness to the book.

Besides these four chapters, the book is outstanding for the subjects treated in the second and last chapters — music, language and meaning, and music, culture and biology, respectively. The second chapter draws together some of the recent thinking on the formal similarities between music and language, and considers the psychological processes that they may share. The last chapter, which looks at the psychological issues raised by cross-cultural studies of music, and examines the neurological and broader biological bases for music, is an important reminder of how ethnocentric most work on the psychology of music is, and of the insights to be gained by widening the cultural perspective.

There are some weaknesses: the comparison of the work of the linguist Chomsky and the musicologist Schenker is too simplistic; the discussion of musical meaning is taken less far than it might have been; and a consideration of the psychological questions stemming from developments in contemporary music would have been welcome. But the clarity of Sloboda's writing and his numerous suggestions for further research will make his book essential reading for anyone, student or researcher, interested in how minds and music interact. □

Eric Clarke is a Lecturer in the Department of Music at the City University, London.

### Fresh information

• *Biotechnology Engineers: Biographical Directory*, edited by O. R. Zaborsky and D. K. Zubris, containing details of 236 "key individuals" in North America. Publisher is Omec International, Washington, DC, price is \$29.95.

• *International Directory of Contract Laboratories* (in toxicology), compiled by Edward M. Jackson. Publisher is Marcel Dekker, price is \$49.75 (North America), \$59.50 (elsewhere).

# New Technology in Biotechnology

*The Nature conference under the above title takes place in London on 25–27 June. Alongside the conference is an exhibition of laboratory equipment.*

## Exhibitors and stand numbers

1. Alfa Laval
2. V.A. Howe
3. Anachem
4. ISC
5. Du Pont
6. Nature
7. New Brunswick
8. & 17. Wisepress
9. & 10. L.H. Fermentation
11. Flow Laboratories
12. S.G.I.
13. Roth
14. Lab Mate
15. Krüss
16. Cambridge University Press
17. Wisepress
18. Bioengineering
19. AMS
20. Uniscience
21. University of Surrey
22. Heraeus
23. & 24. Commonwealth Agricultural Bureaux
25. Leicester Biocentre
26. Anglian Biotechnology
27. Becton Dickinson UK
28. Bactomatic
29. Celltech
30. Apcl
31. Elsevier
32. & 33. Applied Biosystems
34. MBR Bioreactor AG
35. British Library
36. Carl Zeiss Jena
37. Hobsons
38. LEEC
39. Janssen
40. Amicon

*The three-day conference and exhibition take place in the Novotel, Hammersmith. Details of the exhibits on each stand are given in the exhibition catalogue.*

● The Commonwealth Mycological Institute (CMI) is one of four institutes and ten bureaux of the Commonwealth Agricultural Bureaux. It offers a range of services to biotechnologists, as well as mycologists and specialists in related disciplines. A key area of the institute's activities lies in consultancies and contract research. With some 300,000 reference specimens of microfungi (plus 11,000 in the culture collection), a substantial library, online database, and access to electron microscopes, computer and chemotaxonomic and biochemical facilities, the institute is well-placed to help solve problems involving microfungi. In addition, support from the Department of Trade and Industry to the culture collection and industrial services enables the institute to provide new and improved services to industry, and to build up biochemical data on strains held.

*Reader Service No. 100.*

● New Brunswick Scientific has developed a completely new series of pilot-scale fermentation systems designed for mammalian cell culture in bulk suspension and using microcarriers, and for bacterial cell culture. Of particular interest to users of pilot-scale fermenters are the design features in the standard open frame skid concept used by all NBS systems: all piping including filters, valves, regulators and steam traps is housed within the open frame skid, which makes installation and maintenance simple and trouble-free. At the laboratory scale, New Brunswick Scientific will be showing a new fermenter for shear-sensitive cultures, which uses a rotating lift tube for agitation, reducing shear forces on the culture to a minimum. A full range of monitoring and control instruments is available to complement both pilot and laboratory-scale fermentation systems. New Brunswick has recently acquired Biosearch, a company which manufactures a complete range of instruments and chemicals for the synthesis of oligonucleotides and peptides. Both the DNA and peptide synthesizers are fully automated, allowing researchers previously inexperienced in organic chemistry to successfully synthesize the most complex sequences. The instrument software ensures optimum conditions for each coupling once the sequence has been entered via the user-friendly keyboard.

*Reader Service No. 101.*

● The Bactometer, on the Bactomatic stand, is capable of handling 1–512 samples simultaneously, and performs total and selective counts in the range of 1 to 10 million organisms automatically, without the need for tedious procedures such as serial dilutions and plate counts. Results are typically available in 1–8 h. Based on impedance technology, the computer-assisted data system can display impedance growth curves which have been applied to optimization of a fermenter media phase sensitivity and culture metabolism studies, antibiotic/preservative/vitamin assays and sterility testing. Also on the Bactomatic stand is the Vitale AMS system for the rapid, automated identification of microorganisms. The computer-assisted system can identify between 1 and 240 organisms, typically within 4–10 h. The in-built database covers Gram-negative and Gram-positive bacteria, common pathogens and yeast, and can also determine MIC values and sensitivity for up to nine antimicrobial agents simultaneously.

*Reader Service No. 102.*

● Roth Scientific is marketing a series of versatile bench-top fermenters, engineered by Applikon of Holland. A feature of the systems is simplicity of use. Pilot-scale units are similarly available in volumes of 201 to 300 litres. Applikon offers a choice of borosilicate glass fermenter vessels: pilot vessels for bacterial culture have a ratio of height to diameter of 3:1 while those for animal/plant work are shorter and wider, with a 2:1 ratio. The range of vessels includes flat-bottomed, round-bottomed (supported in a tripod) and jacketed vessels all of which can be used to successfully grow bacteria, filamentous organisms, and plant and animal cells. Two forms of stirrer assembly can be specified — a mechanically driven lipseal assembly or a powerful magnetically coupled stirrer which remains coupled even at high speed or high viscosity. Other products offered by Roth Scientific in the Biotechnology range are a versatile ultrasonic disintegrator, which is capable of processing four samples simultaneously, complete high-pressure liquid chromatography systems and a range of freezers and thermostatic baths.

*Reader Service No. 103.*

● Applied Biosystems has chosen the *Nature* exhibition for the launch of its new UK subsidiary, Applied Biosystems Ltd. Among the facilities available are technical support and service laboratories, substantial stocks of reagents and solvents, and a specialist service for protein sequencing, peptide synthesis and DNA synthesis. At the exhibition, there will be on display three new products: the Model 120A PTH Analyser, a specially designed chromatography system for use either 'on-line' with the Model 470A gas-phase protein sequencer (also be on display) or as a 'stand-alone' system for the analysis of phenylthiohydantoin (PTH) amino acids. All common PTH amino acids are resolved to better than 95%, with detection sensitivity of less than 1 picomole. The fully automated Model 381A DNA synthesizer produces high-purity, defined-sequence oligonucleotides quickly and economically, with routine coupling yields of 98–100% and synthesis of oligonucleotides with over 100 bases. The Model 380B DNA synthesizer has been developed as a successor to the 380A and has a new 'touch-screen' CRT control and built-in programming to allow the use of any synthesis strategy. Also on display will be the Model 430A solid-phase peptide synthesizer.

*Reader Service No. 104.*



● Bioengineering UK Ltd will be exhibiting a range of fermentation reactors which includes two new products: the paddle stirrer and visual safety reactor. The paddle stirrer is an agitation system which has particular application in the cultivation of delicate cells. It can be used to convert a conventional stirred tank into a delicate cell reactor. The paddle stirrer system provides a novel solution to the problem of achieving a compromise between agitation effect and dissolved oxygen transfer, using an inversion of a rotational motion which simultaneously disperses and homogenizes. The stirrer transmits its energy mainly by

impulse without shear and burbling. The visual safety reactor, or foil reactor, uses a foil cylinder of high mechanical strength in place of an equivalent glass cylinder. The advantage of the new reactor is that it eliminates the risk of accident when sterilizing glass vessels *in situ*. Visibility is better than with glass, and different foils can be experimented with to obtain novel configurations. The foil can be disposed of after use, thus eliminating the need for extensive cleaning. This concept also allows inexpensive development of 'tower'-type reactors and experimentation with air-lift systems.

Reader Service No. 105.

## ADVERTISEMENT

# How to avoid the 19 critical mistakes in buying fermentation equipment

Make one mistake and you can have problems that include inadequate process control, permanent operator inconvenience and costly downtime.

Now NBS has published a comprehensive review of the problems and pitfalls that await the unwary buyer of industrial fermentors — and some very practical suggestions to help avoid them.

Called *19 Critical Mistakes Made in Buying Fermentation Equipment and What You Can Do to Avoid Them*, this practical guide is available without charge.

Send for your free copy today. New Brunswick Scientific Co., Inc., P.O. Box 986, Edison, NJ 08818. (201) 287-1200 or (800) 631-5417.



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**19  
CRITICAL  
MISTAKES**  
made in buying  
fermentation  
equipment  
and  
what you  
can do to  
avoid  
them

Avoid These 19 Critical Mistakes  
When Buying Fermentation Equipment

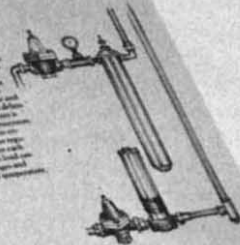
## 1. Lack of a Recirculating System

Equipment that is not equipped with a recirculating system is a major mistake. A recirculating system is essential for the proper operation of a fermentor. It allows the culture to be mixed and aerated, and it ensures that the culture is evenly distributed throughout the fermentor. Without a recirculating system, the culture will settle at the bottom of the fermentor, and the top of the fermentor will be empty. This will result in a poor quality product.



## 2. Failure to Provide Pressure Regulators and Filters

Many fermentors are designed to operate at pressures above atmospheric pressure. If the fermentor is not equipped with pressure regulators and filters, the culture will be exposed to atmospheric pressure, which will result in a poor quality product. Pressure regulators and filters are essential for the proper operation of a fermentor. They ensure that the culture is kept at the correct pressure and that the culture is free from contaminants.



● The Celltech stand features a range of immunoprocessing reagents based on monoclonal antibodies for the purification of  $\alpha$ ,  $\gamma$  and, most recently,  $\beta$ -interferon. Each kit provides single-step, high-yield purification. Celltech's range of monoclonal-based assays includes a gamma immunoradiometric assay (IRMA) for the assay of  $\gamma$ -interferon and NK2 and YOK IRMA for assay of different subtypes of  $\alpha$ -interferon. *Abstracts in BioCommerce (ABC)*, a commercial news index produced twice monthly by Celltech, is now available as an online computer database. At the Celltech stand the database will be demonstrated in action.

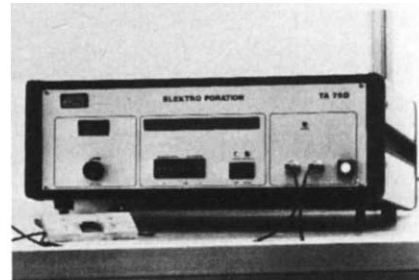
Reader Service No. 106.

● A new 2-litre airlift fermenter that will be ideal for monoclonal antibody production, media optimization and other similar applications is available from LH Fermentation. Based on a 2-litre total volume glass vessel, the fermenter has a working volume of 1,700ml with a removable glass draught tube and a stainless-steel top plate. This has ports for oxygen and carbon dioxide electrodes and other sensors. Temperature control is via thermostatically controlled water circulated around a jacket on the glass vessel; agitation is achieved on the standard unit by the 'airlift' principle with a glass sinter providing the gas dispersion. The LH Fermentation stand will also feature a new 30-litre airlift fermenter which is fitted with complete instrumentation and a gas blending system which can accommodate the culture of shear-sensitive animal and plant cells. And LH's new microprocessor-based fermentation control package can monitor eight parameters in single or multiple fermentation.

Reader Service No. 107.

● EBIP, the European Biotechnology Information Project, based at the British Library in London, and run with the help of European Commission funding, is investigating information provision in biotechnology across a broad front. It has produced a wide range of guides to information sources in key areas such as safety and regulations, business information and culture collections. EBIP is backed by the full resources of the British Library, and EBIP services are also available via the Online system DIMDI.

Reader Service No. 108.



The Krüss TA 750 'electroproration' apparatus for the transfer of DNA and other molecules through cell membranes. Reader Service No. 109.



The Heraeus B5060 cell culture incubator.

● A new, sophisticated cell culture incubator, the electronically-controlled Heraeus B5061 'Auto-Zero', will be displayed on the Heraeus Equipment stand. The CO<sub>2</sub> control on the B5061 has an automatic self-adjusting measuring system to keep the operating parameters constant and reproducible, making manual recalibration of the CO<sub>2</sub> zero unnecessary. This is particularly helpful when the incubator is used for long-term cultivation and manufacturing. Additionally, in the O<sub>2</sub> version, the O<sub>2</sub> zero can be calibrated without interrupting the operation of the incubator. Also on show will be the Heraeus B5060 incubator which, like the B5061, is available with linings made in a choice of bactericidal and fungicidal copper, or stainless steel. With the CO<sub>2</sub> version of the B5060 the temperature and CO<sub>2</sub> content are adjustable with great accuracy, so that the optimum conditions for cell and tissue culture can be created. It has an electronic temperature controller and an adjustable temperature limit controller with a recording facility. An easily drained water bath provides constantly high humidity.

Reader Service No. 110.

● Laboratory and Electronic Engineering Co (LEEC) will be featuring their automatic CO<sub>2</sub> incubators. The GA3 is the large slimline version of the popular 150-litre model GA2, featuring a single chamber with double doors, and a capacity of 330 litres. Both incubators are designed for techniques of open dish tissue cell culture in which the pH of the medium is maintained in equilibrium with the incubator's CO<sub>2</sub> atmosphere. The incubator automatically controls the CO<sub>2</sub> concentration to a pre-set level, and a percentage CO<sub>2</sub> meter on the control panel monitors the output of a thermal conductivity detector cell which continuously analyses a gas sample from the chamber. LEEC are now offering a sterilization feature as an optional extra on both models. An additional boost heating element raises the chamber air temperature to a pre-set temperature of 90–100°C. Also on display will be a selection of compact incubators, tissue culture roller apparatus and drying cabinets.

Reader Service No. 111.

● Flow Laboratories Ltd will be exhibiting a selection of new and existing products for the following: laminar air flow and filtration, microplate technology and tissue culture and diagnostics. On display will be the complete new range of disposable membrane filters, including minifilters for use with syringes, and single or double capsules for the filtration of larger volumes of liquid; and new models of the Titertek enzyme immunoassay readers the Multiskan MCC/MCC340, a twin reader, with the capacity to read, shake and incubate two plates simultaneously; HTE Multiskan Plus with a bar-coded microplate identification system; and Fluoroskan for fluorimetric enzyme immunoassays. All these microplate readers have a bidirectional computer control facility which enables them to be used for kinetic studies. There is also a new range of viral diagnostic reagents for use in complement fixation, enzyme immunoassay and immunofluorescence techniques. Other products on show include a range of liquid-handling devices such as Titertek digital multichannel pipettes and a selection of disposable plastics, tissue culture media and glassware.

Reader Service No. 112.

● Becton Dickinson will exhibit the Falcon range of disposable tissue culture plastics and related products. All Falcon Labware products, from the smallest Petri dish to the largest roller bottle, are sterilized by gamma irradiation. Products featured will include Primaria, a surface-odified plastic substrate for primary cell culture, and the FAST system for simultaneous screening via ELISA of all 96 wells of a hybridoma culture plate for clones producing antibodies of desired specificity, together with disposable filter units, pipettes, multi-well plates and tubes.

Reader Service No. 113.

● The Cetus Pro/Pette, an instrument for the rapid and accurate processing of microtitre plates, will be the centre of Anachem's display. The machine was developed for general cell culture, hybridoma studies and ELISA applications. Plates can be processed either in rows or columns with tip change between rack movement if required. Single wells may be accessed, allowing transfer of samples from tubes to individual wells. The Pro/Pette can also empty and refill wells for serial dilutions or transfer between plates, again with optional tip change. From Gilson, there will be on display pipettes, dispensers, diluters and automatic liquid handlers with 'robotic arms'. Analytical and preparative HPLC equipment, together with examples of Anachem's range of columns and accessories, will also be shown.

Reader Service No. 114.

These notes are based on information provided by the manufacturers. To obtain further details about these products use the reader service card bound inside the journal.

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Reader Service No.44

##### ACADE DIAGNOSTIC SYSTEMS

S.A./N.V., a company specialising in non-isotopic immunoassay using particle counting, is looking for research groups with access to good quality antisera directed towards clinically important assays who would be free to enter into a development contract and/or provide such antisera in commercial quantities.

If interested, please write with full details to Managing Director, ACADE DIAGNOSTIC SYSTEMS S.A./N.V., Passage de la Vecquée, Box 4100, 1200 Brussels, Belgium. Telephone: 02/762.89.75. Telex: 65867. Replies may be in English, German, French or Dutch.

Reader Service No.69

## LASERS SPECIAL ISSUE

The 25 July issue of *Nature* will feature specially commissioned articles on lasers and laser applications, including V. Letkhov on lasers in biology and medicine, S. D. Smith on optical computers, M. Key on X-ray lasers and the late A. Kaster on the early history of the laser.

The issue will also contain a Product Review section on lasers and related products. Manufacturers are invited to send press releases on products or on novel applications to the address below, to arrive not later than 4 July.

Nature (Product Review),  
4 Little Essex Street,  
London WC2R 3LF, UK.



## June meeting

28-29 June, **Investigation and Belief — 3rd Annual International Conference on the Paranormal**, University College, London, UK (M. Hutchinson, Secretary, CSICOP, UK Branch, 10 Crescent View, Loughton, Essex IG10 4PZ, UK)

## July meetings

8-12 July 1985, **7th International Meeting on NMR Spectroscopy**, Cambridge, UK (Dr J. Gibson, The Royal Society of Chemistry, Burlington House, London W1V 0BN)

## September meetings

1-4 September 1985, **First International Symposium on ESR Dating**, Ube, Japan (Prof. Ikeya, Secretary of the Symposium, Technical College, Yamaguchi University, Tokiwadai, Ube 755, Japan)

2-3 September 1985, **International Symposium on Impact Testing and Performance of Polymeric Materials**, Guildford, UK (Miss Rachael Alexander, Short Courses and Conferences Section, Bureau of Industrial and External Liaison, University of Surrey, Guildford, Surrey GU2 5XH)

3-6 September 1985, **Second International Symposium on the Synthesis and Applications of Isotropically Labelled Compounds**, Missouri, USA (Dr R. Wilk, Symposium Coordinator, School of Pharmacy, 5100 Rockhill Road, Kansas City, MO 64110 USA)

16-19 September 1985, **International Environment and Safety Conference**, Olympia, London, UK (D. Parker, Conference Coordinator, International Environment and Safety, Labmate Limited, Newgate, Sandpit Lane, St Albans, Herts. AL4 0BS, UK)

## October meetings

7-9 October 1985, **International Symposium on Biotechnology in the Food Processing Industry**, Minnesota, USA (Lynette Marten, University of Minnesota, St Paul, Minnesota 55108 USA)

7-10 October 1985, **Electronic Imaging '85 — International Electronic Imaging Exposition and Conference**, Massachusetts, USA (R. Murray, Institute for Graphic Communication, 375 Commonwealth Avenue, Boston, MA 02115, USA)

8-10 October 1985, **International Conference on the Occupational and Environmental Significance of Chemical Carcinogens**, Bologna, Italy (Organising Committee, International Conference on Chemical Carcinogenesis, c/o Istituto di Oncologia, Viale Ercolani, 4/2, 40138 Bologna, Italy)

20-23 October 1985, **Halifax '85-16th Annual Underwater Mining Institute**, Nova Scotia, Canada (H. Joseph, Ocean Mining Division, 355 River Road, Ottawa, Ontario, K1A 0E4)

## November meetings

7-9 November 1985, **International Congress on Neo-Adjuvant Chemotherapy**, Paris, France (SOMPS-1st International Congress on Neo-Adjuvant Chemotherapy, Pavillon Jacquart, Hôpital de la Salpêtrière, 47 Boulevard de L'Hôpital - 75651 Paris, France).

19-21 November 1985, **International Conference on Advanced Welding Systems**, London, (The Conference Office, The Welding Institute, Abington Hall, Abington, Cambridge CB1 6AL, UK).

20-22 November 1985, **International Conference on Nitrates in Water**, Paris, France (G. Martin, Ecole Normale Supérieure de Chimie, 35000 Rennes-Beaulieu, France).

## December meetings

2-4 December 1985, **International Conference on Spacecraft Structures**, Toulouse, France (CNES, Département des Affaires Universitaires 18, avenue Edouard-Belin 31055 Toulouse, France)

3-5 December 1985, **Ninth International Online Information Meeting**, London, UK (J. Mulligan, Learned Information (Europe) Ltd, Besselsleigh Road, Abingdon, Oxford OX13 6LG)

3-6 December 1985, **Fourth International Meeting on Biostereometrics '85**, Cannes, France (J. Prado, Coordinator Central du Congrès, ANRT-Association Nationale de la Recherche Technique, 101, Avenue Raymond Poincaré, 75116 Paris, France)

4-5 December 1985, **International Conference on Electric and Magnetic Fields in Medicine and Biology**, London, UK (C. Dagnall, IEE Press Office, Savoy Place, London WC2R 0BL, UK).

11-13 December 1985, **IFAC Symposium on Modelling and Control of Biotechnological Processes**, Noordwijkerhout, The Netherlands (IFAC/BIC '85 c/o Klvl, PO Box 30424, 2500 GK The Hague, The Netherlands).

## Appointments

**Dr Geoffrey Rivett** has been appointed Senior Medical Officer at the Department of Health and Social Security. He will take charge of Med PCR division (Primary Care, Regional Medical Services) with effect from 1 July 1985.

The Council of the Royal Society has appointed **Dr P.T. Warren** as Executive Secretary of the society, on the retirement of **Dr R.W. Keay**.

**Dr Lewis Roberts**, Director of the UK Atomic Energy Authority's Energy Research Establishment at Harwell, has been appointed the first professor of Risk Assessment at the University of East Anglia, Norwich.

Two appointments have been made to the Medical Research Council with effect from 1 August 1985. **Professor Wing** will be Chairman of the MRC's Neurosciences Board, and **Professor Newsom-Davis**, the present Chairman of the Neurosciences Board, will be an independent scientific member.

Three new members have recently been appointed to the Engineering Council. They are **Mr Norman Holland**, UK Group Standards Manager of Philips Electronic and Associated Industries, **Sir Richard 'Brien**, Chairman of the Engineering Industry Training Board, and **Sir Robert Telford**, Life President of The Marconi Company Ltd.

Loughborough University of Technology has appointed **Professor John Phillips** as Vice-Chancellor from 1 January 1986, in succession to Sir Clifford Butler. Professor Phillips is at present director of research and Wolfson Professor in the Wolfson Institute at the University of Hull.

## Awards

**Professor Gert Utermann** from the Institute for Medical Biology and Genetics of the University of Innsbruck has been awarded the Morgagni Medal for his investigations on the pathophysiology of familial hyperlipidaemias. The G.B. Morgagni Young Investigator Awards will be awarded to Dr G. Bolli of the University of Perugia and Dr E. Van Obberghen of the University of Nice.

The William Hopkins Prize of the Cambridge Philosophical Society has been awarded to Dr D.O. Gough of Churchill College for his pioneering achievements in the subject of helioseismology.

The University of Glasgow has appointed **Dr David W.T. Crompton** to the John Graham Kerr Chair of Zoology with effect from 1 October 1985. He is presently lecturer in parasitology in the University of Cambridge, fellow of Sidney Sussex College, Cambridge and adjunct professor in the division of nutritional sciences, Cornell University, New York.

The International Association of Gerontology has awarded the 1985 Sandoz Prize jointly to Sir Martin Roth, Professor of Psychiatry at Cambridge (UK), and Dr Karl Esser, Professor of Botany at the University of the Ruhr (FRG).

The Jung Foundation for Science and Research 1985 prize for Medicine has been awarded to three recipients. The prizewinners are: **Professor Hendrik Coenraad Hemker** — for work on the biochemistry of blood anticoagulation, **Professor Rudolph Pilchmayer** — for work on liver transplantation and **Professor Peter K. Vogt** — for work on the genetic analysis of retroviruses.

Please send 'Announcements' entries to: The Editor, Nature, 4, Little Essex Street, London WC2R 3LF, UK